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THE CONFIGURATION OF COBALTODIHISTIDINE AND OXY-BIS(COBALTODIHISTIDINE) [1] [2]

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INTRODUCTION

It has been shown [3] that a co-ordination complex formed between histidine and cobaltous ion possesses the ability to combine reversibly with molecular oxygen. In contrast with this, histidine complexes with other transition metals tested (Cu^{++}, Cu^{+}, Ni^{++}, Fe^{++}, Mn^{++}) failed to exhibit the property of combining reversibly with oxygen gas.

Although the cobaltous complexes of other amino acids could be demonstrated to form a reversible complex with oxygen, these complexes were in general not so stable and the tendency of other amino acids to combine with Co^{++} was far less than that of histidine. The histidine system is thus conspicuous in two respects: the affinity of histidine for Co^{++} is sufficiently great that complete co-ordination may be obtained at reasonable concentration and pH; the tendency of the histidine-cobalt complex to combine with molecular oxygen is marked and the reversibly oxygenated complex uniquely stable.

The properties which the histidine-cobalt system has in common with hemoglobin and hemocyanin are obvious in that the system consists of a central ion and co-ordinating base and in aqueous solution combines reversibly with oxygen. In view of certain differences, the oxy-bis (cobaltodihistidine) system is, of course, not an exact model for the naturally occurring oxyhemoglobin and oxyhemocyanin. However, the system is clearly of interest because of its reproduction of the most striking feature of the all-important respiratory pigments, and the implications which it holds for similar possible biologically important complexes. It scarcely seems necessary to stress here that knowledge derived from the study of such systems may be expected to implement the rationalization of the properties that render the more complicated systems specific and essential in the wide range of cellular types in which they occur.

In cellular respiration, the privilege of interaction with molecular oxygen is the monopoly of a highly restricted group of catalysts, and the ability to "carry" reversibly and stably bound oxygen gas resides with a still more limited number. In considering the iron-porphyrin complexes, it has become popular, in order to emphasize differences, to regard those reactions in which the state of the metal is raised ($Fe^{++} \rightarrow Fe^{+++}$) as fundamentally distinct from the reversible oxygenation of hemoglobin. Considered from the kinetic point of view, this distinction, otherwise useful, loses its significance. Apparently with cytochrome oxidase, for example, even as in hemoglobin, a reversible oxygen complex occurs, as originally indicated in the work of Warburg (*2*). Assumptions to the contrary leave the well-known problem of carbon monoxide-oxygen competition without meaning. Thus the reversible complexes in the cases of cytochrome oxidase and hemoglobin differ most prominently in stability, e. g., half life, rather than in type. From this it may be concluded that studies of the metal-base-oxygen complexes are of significance not only with respect to their more obvious biochemical counterpart hemoglobin but also with respect to the autoxidizable iron-porphyrin respiratory catalysts and perhaps metal-activated oxidases in general.

[1] Received for publication January 21, 1947.

[2] Since this paper was submitted for publication the following pertinent articles have appeared:

(1) BURK, D., HEARON, J., LEVY, H., and SCHADE, A. L.: Reversible oxygenation of cobaltodihistidine to oxy-bis (cobaltodihistidine), and comparisons, with other metal-amino acids, oxyhemoglobin and oxyhemocyanin. Fed. Proc. 6: 242 (1947).

(2) HEARON, J.: The tetrahedral configuration of cobaltodihistidine. Fed. Proc. 6: 259–260 (1947).

(3) HEARON, J.: The configuration of oxy-bis (cobaltodihistidine). Fed. Proc. 6: 260 (1947).

(4) MICHAELIS, L.: The magnetic properties of the cobalt-histidine complex before and after oxygenation. Arch. Biochem. 14: 17–21 (1947).

(5) BURK, D., FIALA, S., HEARON, J., HESSELBACH, M., LEVY, L., and SCHADE, A. L.: Reversible oxygenation, irreversible oxidation, and decarboxylation of cobalt-amino acid complexes with oxygen gas. Fed. Proc. 7: 342 (1948).

(6) FIALA, S.: Oxygenace a oxygenaceni modely. Biologicke Listy 28: 201–208 (1947).

(7) MICHAELIS, L.: Molecular oxygen as a ligand in metal complex compounds. 113th meeting, Am. Chem. Soc., Chicago, 1948, pp. 32C–33C.

(8) BURK, D., HEARON, J., FIALA, S., SCHADE, A. L., and LEVY, H.: Cobaltodihistidine and related compounds and their significance in oxygenation of metalloproteins. 113th meeting, Am. Chem. Soc., Chicago, 1948, pp. 340–350.

(9) MICHAELIS, L.: Another case of reversible oxygenation. Arch. Biochem. 17: 201–203 (1948).

[3] HEARON, J. Z., BURK, D., and SCHADE, A. L.: Unpublished manuscript.

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In this paper are discussed in a degree of detail not practial in the previously mentioned publications [4] (*1*) the chemical structure of the cobalt-histidine and cobalt-histidine-oxygen complexes and the properties of the constituent metal and base from the standpoint of factors which seemingly confer the observed stability and specificity upon these complexes. It is reasonable to hope that the conclusions drawn, although not necessarily directly applicable, may aid in the delineation of the role of electronic structure in biologic specificity.

ELEMENTS OF THE SYSTEM

Initial points of consideration are that two molecules of histidine combine with one Co^{++}, yielding a complex that is formed immediately and reversibly. Equilibrium studies [5] (*1*) show that the reaction may be written as

$$2\ \text{Histidine} + Co^{++} \rightleftarrows Co(\text{histidine})_2 + 2H^+; \qquad (1)$$
$$K_{25°C.} = (Co(\text{histidine})_2)\ (H^+)^2/(\text{histidine})^2(Co^{++}) = 2\times10^{-7};\ \Delta H = 19.1\ \text{Kcal/mol.}$$

By reaction with oxygen,

$$2\ Co(\text{histidine})_2 + O_2 \rightleftarrows [Co(\text{histidine})_2]_2O_2; \qquad (2)$$
$$K_{25°C.} = ([Co(\text{histidine})_2]_2O_2)/(Co(\text{histidine})_2)^2(O_2) = 3.6\times10^6\ \text{l.}^2/\text{mols}^2;\ \Delta H = -38.2\ \text{Kcal/mol.}$$

Among the naturally occurring amino acids, histidine is characterized by the moderately basic, unsaturated imidazole ring. The resonance in this ring (a subject treated later) has been discussed by Hill and Branch (*3*); suffice to say here that by virtue of the alpha-amino group and the basic imidazole nitrogen, histidine can function as a bidentate chelate in complex formation.

Cobalt, with the other metals of the first transition series, exhibits properties due in large part to incompletely filled *d* orbitals. In these elements the 3*d* orbitals occupy about the same energy level as the 4*s* and 4*p* orbitals (*4*, *5*) and play a major role in bond formation. Of importance in this group of elements are the possible ways of filling the available orbitals. The available orbitals are made clear in the following diagram: from the electronic distribution of cobalt, (A)$3d^7 4s^2$, and Hund's rule, Co^{++} is pictured as

$$\underline{(oo)(oo)(o\ )(o\ )(o\ )}_{3d}\ \ \underline{(\ )}_{4s}\ \ \underline{(\ )(\ )(\ )}_{4p},$$ and for

the purpose of comparison,

$$Ni^{++}\ \text{as}\ \underline{(oo)(oo)(oo)(o\ )(o\ )}_{3d}\ \ \underline{(\ )}_{4s}\ \ \underline{(\ )(\ )(\ )}_{4p},$$

and Cu^{++} as

$$\underline{(oo)(oo)(oo)(oo)(o\ )}_{3d}\ \ \underline{(\ )}_{4s}\ \ \underline{(\ )(\ )(\ )}_{4p}.$$ These

three ions show a tendency to assume the nearest inert-gas structure (krypton). This is possible by the gain of 11, 10, and 9 electrons for Co^{++}, Ni^{++}, and Cu^{++}, respectively. Since each ion forms a 2:1 complex with histidine, it may be considered that 8 electrons are derived from 4 nitrogen atoms, and the krypton structure would be achieved by the further gain of 3, 2, and 1 electron, respectively. It is a matter of experience that the ions of the first transitional series usually exhibit a co-ordination number of 4 or 6. It is of value now to consider the ways in which the available orbitals may be combined to form stable bonds, and the number of such bonds permitted.

DIRECTED COVALENCE

Pauling (*5*, *6*, *7*) and Slater (*8*) have treated the question of directed valence from the quantum-mechanic point of view. This treatment has been extended by Hultgren (*9*) and Kimball (*4*, *10*). In particular, Kimball has investigated the possibilities of double-bond formation. These results give the geometrical configuration obtained when specific orbital combinations (bond orbital hybridization) are employed. Rules for the occurrence of these configurations and "bond strength" are also given. In the theory of directive bonds of Pauling and Slater, the bonds formed by a given atom tend to assume certain preferred directions relative to each other. Specifically, the bond formed by a given orbital tends to lie in the direction in which the orbital is concentrated. The bond strength is a measure of the extent to which the orbital in an atom will "overlap" with the orbital of another atom. Further, with certain limitations, these results make it possible to relate magnetic properties and configuration.

The relevant rules may be summarized as follows: In the iron group, only the 3*d*, 4*s*, and 4*p* orbitals are used in bond formation. When one *s* and three *p* orbitals are used in the hybridization process, the result is four equivalent tetrahedral bonds. Thus the sp^3 hybridization is a regular tetrahedron; the bond strength is 2.000. By the term "equivalent bonds" is meant bonds which differ only by rotation. Similarly, when one *d*, one *s*, and two *p* orbitals are used (dsp^2), the result is four equivalent bonds which lie in a plane and are directed towards the corners of a square; the bond strength is 2.694. This is the configuration expected when only one *d* orbital is available, for these are the strongest bonds that can be formed, and under these conditions, no more than four strong bonds may be formed. In the following

[4] See footnote 3

tabulation, the configurations of interest in this discussion are taken from Kimball (*10*), and the bond strengths from Pauling (*6, 7*) and Hultgren (*9*) when available.

Orbital	Configuration	Bond strength
sp^3	Regular tetrahedron	2.000
d^2sp	Irregular tetrahedron	-----
dp^3	Irregular tetrahedron	-----
dsp^2	Square	2.694
d^2p^2	Tetragonal plane	-----
d^2sp^3	Octahedron	2.923

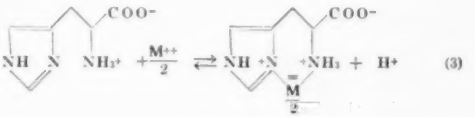
The foregoing results have at least two important limitations: (1) They apply to covalent or predominantly covalent bonds, and when ionic contributions are large, predictions may not be closely valid; and (2) group interaction may cause bond distortion, and the resultant molecule will be a forced configuration of minimum potential energy.

In the cases of the three ions in question, it is to be expected that covalent Ni^{++} and Cu^{++} complexes will be planar (square). Evidence for the square configuration has been provided by the magnetic properties of many nickel complexes, and detailed verification furnished by cis-trans-synthesis, X-ray study, and dipole moments (*5, 11*). On the other hand, it should be mentioned that a number of nickel complexes are paramagnetic. In these complexes, four groups are presumably arranged tetrahedrally about the central ion; this has not been demonstrated by other means, and the evidence for tetrahedral Ni^{++} is regarded by some as meager (*11*). It is not possible to state what factors determine whether the diamagnetic or paramagnetic configuration will be assumed by Ni^{++}; but from an examination of the literature it appears that when nitrogen or oxygen atoms are involved, the presence of double bonds plays a role. There are exceptions to this generalization (*11*), but the possibilities of double bonds are greater for the dsp^2 configuration than for the sp^3 (*10*). Cases are recorded (*12*) in which a given compound of nickel is diamagnetic under one set of conditions and paramagnetic under other, fairly similar conditions. The difference in stability of the two configurations must, at least in these cases, be small. The important consideration for our purpose is, however, that when, as in Ni^{++}, only one d orbital is available, no more than four strong bonds may be formed.

When the orbital arrangement for Cu^{++} is considered, it is seen that one d orbital is available only if the promotion of one electron is effected. Such a promotion, entirely reasonable from the energetic point of view, enables the planar dsp^2 configuration to be assumed (bond strength 2.694) in preference to the tetrahedron sp^3 (bond strength 2.000). The planar configuration is apparently universal for the Cu^{++} complexes; and although the magnetic criterion is not applicable, all cases investigated from the standpoint of crystal structure and isomerism are planar. The Cu^{++} and Ni^{++} protoporphyrins exhibit absorption bonds which have been correlated with strong covalent bonds in such complexes (*13*).

The orbital arrangement in Co^{++}, with three single electrons in $3d$, presents several alternatives. From an investigation of a series of complexes, Mellor and Goldacre (*14*) stated that the tendency is for Co^{++} to form essentially ionic tetrahedral or octahedral complexes. It is frequently stated (*11, 15, 16*) that a magnetic moment corresponding to one unpaired electron indicates the square structure dsp^2 in cobaltous complexes. That this is not necessarily the case is seen at once from the possible configurations worked out by Kimball (*10*); the tetrahedrons d^2sp and dp^3 would both give one unpaired electron. Recently, however, Calvin and others (*17*) reported on complexes of Co^{++} with one unpaired spin which were definitely shown by X-ray analysis to be planar.

The general reaction between histidine and any of the three ions under consideration may be written as



(3)

For Co^{++} it is expected that the d^2sp tetrahedron would be formed, giving the distribution $3d^{10}4s^23p^3$ with a magnetic moment of 1.73 Bohr magnetons, unless the complex is ionic, in which case the value would be 3.88; for Ni^{++} and Cu^{++}, the dsp^2 plane is expected, with the distributions $3d^{10}4s^24p^4$ and $3d^{10}4s^24p^5$, respectively, the Ni^{++} being diamagnetic if covalent and the Cu^{++} complex showing a moment of 1.73 Bohr magnetons. Stress is placed upon the fact that, of these complexes, the Co^{++} complex would be expected to take up readily the d^2sp^3 octahedral structure by acquiring further groups, while the other complexes should show little if any such tendency. Discussion of the charge distributions in these complexes is given later.

CO-ORDINATION WITH MOLECULAR OXYGEN

The co-ordination of the Co^{++}-histidine complex with molecular oxygen presents several possibilities which must be evaluated. Each possibility must conform to the equilibrium data which demand the general stoichiometric formulation shown in equation 1. Thus the O_2 molecule must be placed between, or at least bonded to, each of two complex molecules occupying one or more co-ordinate positions on each Co^{++}. Within these

conditions of constraint exist the possibilities considered herein.

The normal state of the O_2 molecule is generally accepted to be

If no change is induced in the O_2 structure upon co-ordination, one may picture the oxygenated complex as

A

in which the bond orbitals would be the d^2sp^3 octahedron with the distribution $3d^{10}4s^24p^65s^1$. The expected magnetic moment would be 2.83 Bohr magnetons per Co^{++}. If pairing occurs within the O_2 molecule itself, the same octahedral bonds and electron distribution would hold for the complex

B

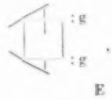
but the magnetic moment would drop to 1.73 Bohr magnetons per Co^{++}, this being due to the unpaired spin of one electron in each $5s$ orbital of Co^{++}. Entirely analogous structures may be formulated with each of the O atoms occupying one position on each Co^{++}, i. e.,

C

and

D

where g is a group as yet unspecified. As a matter of fact, it would be impossible to deny that in solution, the complex was, in the absence of O_2, the octahedron



E

where g may be water or carboxyl; it is reasonable to assume that such groups as would be available, especially water, would be bound by electrostatic or ion-dipole bonds, and one or both groups easily replaced for a covalent bond from O_2. This would raise the question why only one of two equivalent groups is displaced by O_2 if C or D is formed. It is possible that steric considerations would rule out a second O_2 molecule. Similar conditions, however, would apply to H_2O, for the covalent radius of O in H_2O is 0.66Å, and the H–O distance 0.31 Å, while the octahedral covalent radius of Co^{++} is 1.32 Å; but it is certain that the H_2O:Co^{++} bond would be ion-dipole, while the stable bonding of O_2 is expected to be through relatively strong covalent bonds.

One striking feature of the Co^{++}–histidine complex is that it does not readily, if ever, become oxidized to the Co^{+++} form in the presence of O_2. On the other hand, a notable characteristic of octahedral Co^{++} complexes is that they are easily autoxidizable. This has been attributed to the promotion of a single electron to the $5s$ orbital. The effect of complex formation and concomitant promotion on the Co^{++}–Co^{+++} equilibrium, and the contrast with similar Fe^{++}–Fe^{+++} systems in which no promotion occurs were discussed by Pauling (*6*, *7*). Thus, the stability of the Co^{++}–histidine complex must be taken into consideration, and any proposed electronic configuration should provide an explanation of this property. The structure

F

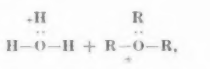
fulfills this requirement. Here the single electron of Co^{++} has not been promoted to $5s$ but is paired in the $4p$ orbital with one of the single electrons in O_2. The distribution would be $3d^{10}4s^24p^6$ (which is recognized as the krypton configuration) and the complex, in contrast with the other possibilities indicated (A–D), would be diamagnetic instead of paramagnetic. The corresponding structure

G

would also fulfill the necessary requirements.

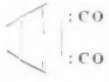
The various possibilities may be compared as follows: In A, each O atom has a covalence of 3 (one normal covalence and two dative or co-ordinate bonds), and the cobalt bears a formal charge of −4. In C, each O atom has a covalence of 2, and the cobalt is formally −4. Similarly in B, each O atom has a covalence of 4, and the cobalt is −4, while in D the O atom has a covalence of 3, and cobalt is −4. In the structures F and G, in which the single electrons of Co^{++} and O_2 are paired, each O atom has a covalence of 3 and 2, respectively, and cobalt has a charge of −3 in each

case. No data yet available allow a conclusive choice between F and G, but it is most probable that one or the other of the structures, in which the single cobalt electron is not promoted but coupled, is to be preferred; further, these formulations assign O a covalency of 3 or 2, both of which are commonly found in the hydronium ion and oxonium compounds,



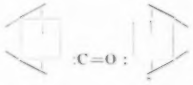
and the true peroxides R-O-O-R; also, the charge on the cobalt, which must surely be distributed throughout the molecule, is least in these two cases.

Cobaltodihistidine does not react with carbon monoxide. This is surprising in view of the fact that alkaline Co^{++} will take up CO (*18*), that CO decomposes Co^{++}-cysteine complexes (*19*) and enters Fe^{++}-cysteine complexes. It is somewhat difficult to understand why no CO combination with cobaltodihistidine was observed since a number of possibilities are seemingly open, such as



similar to $Co(CO)_4$, or

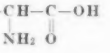
similar to the iron carbonyl $Fe_2(CO)_9$ (*6*) or



While several reasonable possibilities such as these may be written, it is impossible to write a plausible CO complex analogous to the F complex containing O_2. It is known that at least one other tetraco-ordinated cobaltous complex, viz, Co^{++}-protoporphyrin (*20*) does not react with CO.

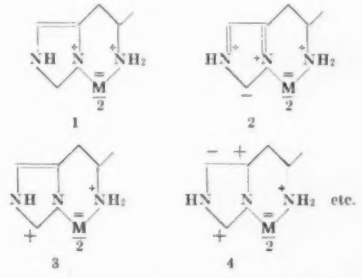
CHARGE DISTRIBUTION, RESONANCE AND CONJUGATION

The question of charge distribution in the Co^{++}-, Ni^{++}-, and Cu^{++}-histidine complexes leads to a discussion which is also of value in attempting to explain the effectiveness of histidine, as compared with other amino acids, in binding Co^{++} or other metals. In all cases, histidine was characterized by being far more effective than any other amino acid except cysteine. (Where a 3:1 stoichiometry with Co^{++} was observed, and still greater affinity, without formation of a reversible O_2 complex, however.) We must look then to the co-ordinating base for an explanation of the stability of the histidine complex. In a fairly general way it may be stated that the basicity (co-ordination with H^+) of an atom, in particular N, parallels its tendency to co-ordinate with metals; that is to say, the "availability" of the two dative (2*s*) electrons determines the strength of the co-ordinate bond so far as N is concerned. In cases in which this correlation breaks down, other factors must enter. One such factor of great importance was brought out by Calvin and Bailes (*21*), namely, if a given number of groups co-ordinates with a central ion, the resultant complex is more stable if the groups are bonded together, e. g., 6 NH_3, about an ion would be less stable than 3 $NH_2—CH_2—CH_2—NH_2$ about the same ion. This factor is certainly present in the case of histidine, the amino group and ring N being in the same molecule. While spatially this bonding between the two functional groups may be less favorable in other alpha-amino acids (which presumably co-ordinate through carboxyl and amino groups (*22*, *23*)), it is also present in them:



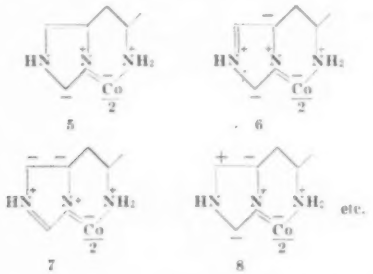
Further, the pK values of the amino groups of the various amino acids are not greatly different. We must, therefore, inquire into other influential factors which determine the stability of these metal complexes, in particular such possibilities as are not open to other amino acids but are available to, preferably peculiar to, histidine.

Resonance possibilities in the ring structure of histidine are immediately suggested. Taking representative forms of the imidazole ring, we write

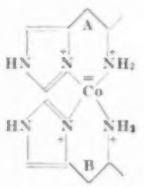


In none of these is the nitrogen bond directly affected, but it may be affected indirectly by polar or inductive effects. The full possibilities of double-bond formation may be utilized by forming a double nitrogen-metal bond, provided that there are available orbitals which are permissible for geometrical considerations. Double-

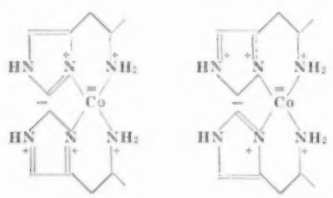
bond formation in covalent complexes has been definitely indicated (*5*, *24*), and Kimball (*10*) shows that a specified number of double bonds is geometrically possible for each configuration. For the tetrahedral cobaltodihistidine one *d* orbital is available for such bond formation. Thus it is possible to write



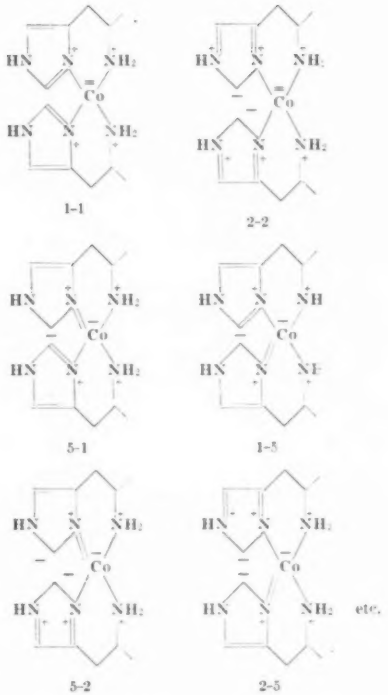
Since only one double bond may be formed to the cobalt atom, if one cobalt containing ring is in a double-bonded form, the other ring must be in a form which excludes the double nitrogen-cobalt bond; that is, in:



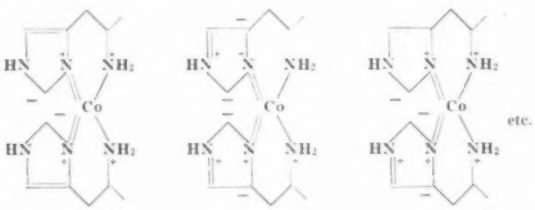
if ring A is in one of the forms 5, 6, or 7, etc., ring B must be in one of the forms 1, 2, or 3, etc. It is expected that this resonance is less effective because of the foregoing restriction, an effect similar to cross conjugation (*25*, *26*) i. e., when two resonances are mutually exclusive, the effectiveness of both is decreased. If 1 represents the groups of type 1, 3, 4, etc., and 5 represents the group of double-bonded cobalt forms, 5–8, maintaining 2 distinct, since it is a form in which a bond in the cobalt-containing ring, other than the nitrogen-cobalt bond may be doubled[6] eight groups of resonance forms can be obtained, illustrated by:



[6] These arbitrary groupings are not on a basis of similar energy structures and 2 represents a group of four similar energy structures. 3 represents a group of five, and 4 a group of seven such structures. The division made here is chosen because of the location of bonds, as becomes clear later.



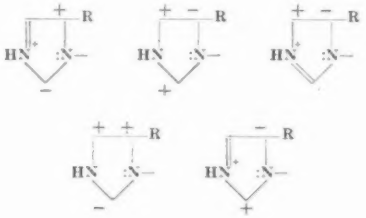
When cobaltodihistidine takes up O_2 and assumes the octahedral configuration, three *d* orbitals are available for double bond formation.[7] As no double can be written involving the amino nitrogen, we can employ only two of these orbitals by doing so, the additional series of structures is possible, such as



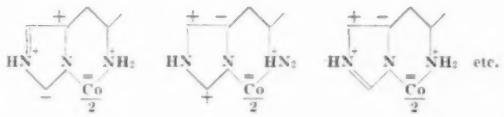
In structures such as 3 and 4, the charge is removed from the cobalt-bound nitrogen. In the structures involving the double bonded cobalt, such as 5–8, the formal charge on the cobalt atom is reduced to −1; and the negative charge, otherwise residing upon the cobalt, is distributed into the imidazole rings. In the forms involving two double bonds to cobalt, 9, 10, 11, etc., the charge

[7] Note that the *d* orbitals available in the octahedron d^2sp^3 may form "strong π bonds" (*11*) whereas the previously available *d* orbital formed only "weak π bonds." Thus, in so far as the possibilities of strong multiple bonds stabilize the complex, the oxygenation of cobaltodihistidine increases the stability.

on the cobalt is still -1 since the coordinate O_2 bond of complex F or the *g* group of G introduces an additional negative charge which is offset by the increased possibility of double-bond formation. A number of forms of the histidine molecule, which would be minor forms, assume more importance in the resonance of the complex. Of the seven possible forms with double-charge separations, all become, in the complex, single-charge separation forms, and the five forms with one or more adjacent charges

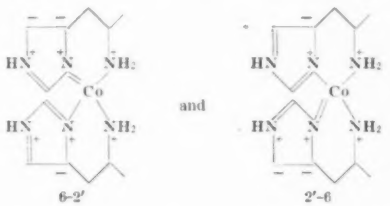


become, in the complex,



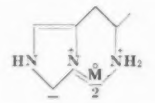
in which two have no adjacent charges and three have one charge adjacency. These forms then should contribute more heavily to the resonance of the complex than to that of histidine itself, and the forms 5–8 are not possible except in the complex. The remaining resonance forms[8] would have about equal weight in histidine and in the complex.

Several forms are possible in which the four double bonds in the complex are fully conjugated, thus



would be two equally contributing, identical energy structures, as would be 5–2 and 2–5 on p. 6. Possible also are pairs such as 5–1 and 2–6, or 2–6 and 5–1, which, however, are not identical energy structures. Such conjugation undoubtedly accounts for the deep color of the oxygenated complex.

In the nickel and copper complexes, three *d* orbitals should be available for double bonds (*10*) and two double bonds to the metal may be formed. Here, however, the charge on the metal is completely removed and distributed into the ring. The stabilizing effect of such charge distribution has been discussed by Pauling (*5*).



Thus, the stability of the histidine complexes may be, at least in large part, explained on the basis of one of the more important specific attributes of the histidine molecule. This stabilizing resonance is evidently not possible for the other amino acids, and the double-bonded structures possible for metal co-ordination may not be expected to contribute to co-ordination with H^+.

Magnetic Properties

Since no great success has attended attempts to obtain crystalline preparations of cobaltodihistidine and oxy-bis (cobaltodihistidine), magnetic susceptibility measurements contribute the most conclusive proof of the predicted configurations.

The magnetic moment of cobaltodihistidine was found to be 4.44 Bohr magnetons. The calculated spin moment for three unpaired electrons is 3.88. The observed value is in good agreement with that usually found in ionic cobaltous complexes with about the same quenching of orbital moment as in the hydrazine complexes, for example, in which there are four nitrogen-Co^{++} bonds. Thus it is concluded that the complex involves bonds which are predominantly ionic in nature. As pointed out (p. 3), when the bonds are essentially ionic, the directed nature of covalent bonds has little importance in determining the arrangement; the position of minimum potential energy for four ionic bonds is, however, the tetrahedral arrangement. The observed moment for oxy-bis(cobaltodihistidine) was zero. From this it is concluded that there are no unpaired spins in the oxygenated complex. This finding is rather definite proof that the structure involves the pairing of a single electron from Co^{++} with one from O_2, as was suggested to explain the stability of the complex and discussed on page 4. In fact, no other formulation than F or G (p. 4) is in accord with the stoichiometry of reaction 1, the stabilization of the cobaltous state, and the observed magnetic

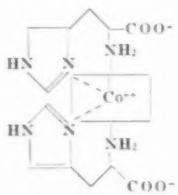
[8] Nine forms in all, four with single charge separations, octets about all atoms; five with single separations, sextet about one atom. Compare reference (*4*).

moment.[9] Furthermore, it is evident that the bonds in the oxygenated complex, which may be stated with some certainty to be the d^2sp^3 octahedral configuration, are predominantly covalent; thus the nature of the bonds has changed upon oxygenation from essentially ionic to essentially covalent. Such a transition in bond nature is observed upon the oxygenation of hemoglobin (*27*).

The "irreversible complex" [10] (formed by the uptake of an additional O_2 which renders the overall reaction irreversible) is diamagnetic. There is little that can be definitely stated at this time regarding the nature of this complex and the manner in which the second O_2 enters the structure. In certain cases, seemingly minor changes vastly alter the stability or reversibility of a complex. For example, NO is expelled by HCl from $[Co(NH_3)_5NO]Cl_2$, which is black and paramagnetic, but not from $[Co(NH_3)_5NO](NO_3)_2$, which is pink and diamagnetic (*28*). Whatever the mode of combination involved with the second O_2 molecule, the paramagnetic O_2 molecule is deprived thereby of its unpaired spins.

Optical Properties

A complete study of the various optical properties of cobaltodihistidine and oxy-bis(cobaltodihistidine) has not been carried out because of the intense color, especially of the latter compound, and the difficulty of working either in the complete absence of oxygen or under a sufficient pressure of oxygen to assure complete oxygenation at the desired temperature and concentration. Satisfactory rotation studies have been made, however, on cobaltodihistidine.[11] It is found that while *l*-histidine, from which the complex is prepared, has a constant rotation throughout the visible range, the complex exhibits marked changes in rotation with varying wavelengths. The sign of the rotation goes through a (negative) minimum, becomes zero within the absorption band, and goes through a (positive) maximum, viz, the Cotton effect is observed. Although the phenomena associated with the Cotton effect are of great theoretical importance, it is not possible at this time to give a complete discussion of these effects in relation to the proposed structure of cobaltodihistidine. The following remarks are, however, relevant. The occurrence of the Cotton effect has previously been put forward as evidence for the tetrahedral configuration; certain cases in which this evidence has been employed have not been well founded (*11, p. 177*), and it seems wise to suggest such evidence in any case only as supplementary evidence. The Cotton effect is exhibited only if the electronic system responsible for the band is also a major contributor to the optical activity; the principles applied by Kuhn (*29*) to the analysis of the Cotton effect apply to, and only to active spiro-compounds in which two rings are set at right angles. The two six-membered rings, referred to on p. 6, are arranged at an angle, presumably of 90°.



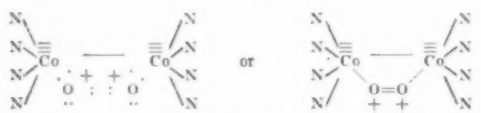
As suggested, the color of the oxygenated compound is probably due in large part to the conjugated bond system. It seems more likely that the color and absorption band of cobaltodihistidine, which band is absent in both Co^{++} and histidine, is due to the contribution of ionic terms. (Compare the shift in spectra of cobaltoamines caused by replacement of NH_3 by Cl.)

The intense color of oxy-bis(cobaltodihistidine) prevented any type of rotation studies. In the case of the irreversible complex, even though the color precluded highly accurate measurements, it could be determined that markedly anomalous dispersion occurred, which was entirely different qualitatively from that of cobaltodihistidine.

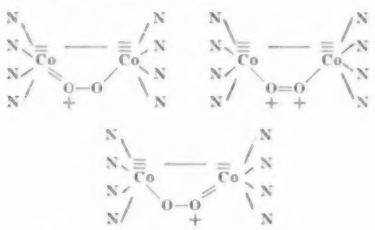
DISCUSSION

It is evident from the foregoing that the data available do not allow a choice between the two most probable structures (F and G, p. 4) for the oxygenated complex. As previously pointed out, these two structures differ chiefly in the formal charge upon the atoms of the O_2 molecule. In F

[9] This statement may be qualified by the interesting suggestion of the possibility that a covalent bond is formed between the two cobalt atoms giving



which would fit the magnetic data and also offer the possibility of a stabilizing resonance involving the O_2 molecule:

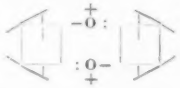


While the Co-Co distance in the metallic bonds (*6, pp. 406–407*) and the oxygen-oxygen distance in O-O are not incompatible with such a suggestion, there is no collateral evidence which suggests a metal-metal bond; and until such evidence is available, the formula F or G is most satisfactory.

[10] [11] See footnote 3.

[12] Se

the rather unsatisfactory feature of adjacent formal charges is present:



It is impossible to distribute this charge or to picture any resonance involving the O_2 molecule without violating the octet rule. G involves the indefiniteness which necessarily accompanies the assignment of group g, which may be any available group.

It is most reasonable to suppose that the carboyxl group is the group in question since it has been shown [12] by comparing cobaltodihistidine and cobaltodihistamine that the presence of the $-COO^-$ enhances the stability of the unoxygenated complex to the extent of 4.1 K cal mol^{-1}. The most logical assumption is that this is due to electrostatic interaction between the $-COO^-$ and Co^{++} or to any "bonding" between $-COO^-$ and Co^{++} in such a manner that positions may be made available for bonding with O_2.

With regard to the suggested structure involving a metallic bond, it may be said that it involves satisfying the possibility of distributing the charge on the oxygen atoms, resonance involving the O_2 molecule, and the possibility of forming the maximum of three double bonds to cobalt, and hence complete removal of charge from the central atom. A similar structure has been suggested (*5*) for diamagnetic $Fe_2(CO)_9$ on the basis of crystal structure (*30*), and rather tentatively suggested for the binuclear ferric-orthophenanthroline complex of Gaines et al. (*31*); however, the occurrence of this type of bond in covalent complexes, especially in aqueous solutions, is not well-established. Although the suggested complex offers interesting and desirable structural features, it is suggested merely as the only additional possibility in accord with the available experimental evidence. The structures F and G represent the most probable structures, and except for the points mentioned, there is little choice between the two.

While the utilization of a single electron from the metal and from O_2 in the formation of the metal-O_2 bond in oxy-bis(cobaltodihistidine) is unlike the corresponding bond in oxyhemoglobin, the change in bond nature upon oxygenation constitutes a striking point of similarity between the two compounds; it is also evident that in both cases the electronic structure of the O_2 molecule undergoes a distinct change. It is not yet possible to state what the importance of this change in multiplicity will prove to be for the characteristic nature of hemoglobin and cobaltodihistidine. The infrequency of such transitions, previously observed only in hemoglobin derivatives, was pointed out by Pauling (*27*). In addition to the two cases mentioned, a number of examples are available to date, namely, formation of the hemochromogens (*13*), formation of the CN^- and SH^- complexes of peroxidases (*32*), formation of the CO complex of ferroperoxidase (*32*), and the oxygenation of two distinct types of O_2 carriers studied by Calvin et al. (*33*). In all cases, the molecules involved are certainly of a specialized nature in that they are iron-porphyrin derivatives, O_2 carriers, or both.

Two significant differences between hemoglobin and cobaltodihistidine are that the latter forms a binuclear complex in which the ratio of metal to oxygen is 2 : 1, and that the cobalt complex, as previously mentioned, does not combine with carbon monoxide. These points, however, immediately suggest an interesting analogy to the hemocyanin-oxyhemocyanin system. Little detailed knowledge is available regarding this copper protein. The valence of the metal is still an open question, and there is serious doubt that the copper in this protein has ever been oxidized or reduced by laboratory methods (*34*). From the data of Rawlinson (*34*) hemocyanin is apparently diamagnetic. It is definite that the metal: O_2 ratio is 2:1 in oxyhemocyanin (*35–37*), and that no compound is formed between CO and hemocyanin (*35*, *38*). Thus, we have the interesting situation that hemoglobin, which forms a complex in which metal: O_2 is 1:1, combines with CO, whereas the two carriers which form binuclear complexes with O_2 do not.

It is interesting to note the role which steric restrictions play in the complex discussed in this paper and other O_2 carriers. With the usual amino acids such as alanine, glycine (*39*, *40*), and cysteine [13] (*41*), it is possible to form complexes involving three amino acids and one cobalt, thereby filling all available positions. With histidine, it is not possible, apparently, because of steric hindrance, for three molecules to surround one Co^{++}; certainly only two are observed to do so.[14] Thus, co-ordinate positions are held open or, as previously discussed, are filled by weakly bonded groups that are easily replaced by O_2. It is believed that in the case of hemoglobin one group, apparently as imidazole of histidine, is held in the globin molecule in a position sterically unfavorable to co-ordination with Fe^{++} and is hence easily replaced by O_2 (*42*). An impressive example of the operation of this principle is afforded also by a series of compounds developed by Calvin et al. (*17*, *33*) in which the chelate-forming compound is capable of filling five of the six positions, leaving one available position for O_2 co-ordination. When NH_3 or CN^- is the complex-forming compound,

[12] See footnote 3.

[13] [14] See footnote 3.

it is usually observed that the maximum number of groups for which strong bonds are available is taken on by the central ion, i. e., the compounds $Co(NH_4)_4$ and $Fe(NH_4)_4$ and $Fe(CN)_4$ have not been observed. Kimball (*10*) points out that such complexes (which would be expected to have the d^2sp configuration) are apparently unstable. It also seems probable that when the size and nature of the groups involved impose no limitation upon the number taken on by the central ion, the reaction is not readily stopped at an intermediate stage, and complexes similar to those involving large polydentate molecules are not obtained.[15] The stabilizing effect of bidentate or polydentate complex formers (*21*) has already been discussed. This factor, together with the above-discussed factor, and the presence of groups which allow stabilizing resonance seem to be structural features common to the O_2 carriers mentioned, and of great importance in determining the properties of these molecules. The bonding together of functional (co-ordination) groups and the accompanying steric restrictions of the resulting larger molecules undoubtedly will ultimately be shown to impose the limits of simplicity upon complexes which will carry O_2. In fact studies of peptides of histidine (which offer the possibility of forming ring systems which more closely approach natural systems) show that at least this type of alteration of the histidine molecule is accompanied by a more rapid irreversible reaction. It might well be concluded from this that cobaltodihistidine represents virtually the most simple attainable molecule combining the desired carrier properties with those of water solubility and the ability to function under essentially physiologic conditions. As such, it represents an excellent model for study as well as a suitable beginning point for further work since its properties may probably be improved only by means entailing the introduction of increased complexity.

SUMMARY

The configuration of cobaltodihistidine and oxy-bis (cobaltodihistidine) are discussed. Factors which determine the unique stability of these structures and which distinguish them from other metal-histidine complexes and other cobalt-amino acid complexes are pointed out. Comparisons are made with the natural carriers, hemoglobin and hemocyanin, and differences and similarities discussed.

The available bond orbitals in Co^{++} are such that stable tetra- and hexaco-ordinated complexes may be formed; the most stable of these is the covalent octahedral (d^2sp^3) complex. On the other hand the available orbitals in Cu^{++} and Ni^{++} (the histidine complexes of which do not carry oxygen) are such (one d orbital available) that only four strong bonds may be formed.

The essential feature of the combination of cobaltodihistidine with molecular oxygen is the pairing of a single electron from Co^{++} with a single electron from the O_2 molecule. It is this pairing of a single Co^{++} electron which accounts for the stabilization of the cobaltous state in the oxygenated complex. No other formulation is in accord with the observed magnetic moments and the stabilization of the bivalent state of the metal.

If the availability of a single electron in the metal is necessary for combination with oxygen it is clearly not sufficient, for a single ($3d$) electron is available in Cu^{++}, the histidine complex of which does not combine with oxygen. The restricting principle in this case appears to be that with one d orbital available only four strong bonds are permitted. This is borne out by the fact that the histidine complex of Ni^{++} does not combine with oxygen.

In cobaltodihistidine positions are held open (or can be readily made available) for combination with oxygen. It is pointed out that this is in contrast with certain nonoxygen-carrying complexes of cobalt. The operation of this type of constraint in hemoglobin and in the oxygen carrying chelates studied by Calvin et al. is pointed out and briefly discussed.

The bonds in cobaltodihistidine are essentially ionic; those in oxy-bis (cobaltodihistidine) strictly covalent. Such a transition in bond nature with the concomitant loss of paramagnetism has been observed to occur in the oxygenation of hemoglobin. From the discussion of the configuration of oxy-bis (cobaltodihistidine) here presented it is clear that the mechanism underlying the disappearance of unpaired spins in the oxygen molecule is fundamentally different from that in the case of oxyhemoglobin.

It is pointed out that the stability of cobaltodihistidine relative to that of the cobaltous complexes of other α-amino acids may be explained on the basis of the presence (and nature) of the imidazole ring in histidine. The effect of stabilizing resonance involving the imidazole ring should be especially prominent in the covalent oxygenated complex in which the maximum number of double bonds between nitrogen and cobalt may be formed. The contribution of such double bonded structures removes the formal charge from the cobalt atom and permits stabilizing charge distribution throughout the imidazole ring. The formulae are presented, and the relative importance of the various resonance species is commented upon. It is obvious that the above-discussed structural features which enhance stability are present only in histidine and histidine derivatives.

[15] Cognizance must be taken, of course, of the importance of the inherent nature of the co-ordinating groups and such manifestations of this as stabilization of valence, etc. That no generalization regarding valence stabilization may yet be drawn has been the subject of a review (*43*), and the matter is not discussed further in the present paper.

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CHEMICAL ALTERATION OF POLYSACCHARIDE FROM *SERRATIA MARCESCENS* I. TUMOR-NECROTIZING POLYSACCHARIDE TAGGED WITH RADIOACTIVE IODINE[1,2,3]

By Arnold M. Seligman, M. J. Shear, Joseph Leiter, and Benjamin Sweet

INTRODUCTION

Following the isolation (*1*), development of an assay method (*2*), purification (*3*), and characterization (*3*, *4*) of the tumor-necrotizing material from cultures of *Serratia marcescens*, the material was administered to patients with inoperable cancer (*5*, *6*, *7*). Severe febrile reactions, followed in some cases by circulatory collapse, were obtained with initial doses varying from 10 to 50 micrograms, which is in the vicinity of the maximum tolerated dose. The mouse, on a weight basis, tolerates much larger doses, viz, more than 1,000 times as much. The maximum initial dose tolerated by patients is, moreover, only about one-tenth the minimum necessary for producing detectable damage in a sensitive mouse sarcoma (*2*). Yet hemorrhage and necrosis were produced in some human tumors, notably sarcoma, even at this low level (*6*, *7*). If the toxicity of this agent could be reduced sufficiently to enable patients to tolerate doses comparable with those effective in tumor-bearing mice (*8*), extensive destruction of tumor tissue might be obtained regularly.

Accordingly, the possibility was explored of reducing the toxicity of this polysaccharide by chemical means without decreasing its tumor-necrotizing properties. This paper deals with the incorporation of radioactive iodine into the polysaccharide molecule. The extent of incorporation and the effect of iodination on tumor-necrotizing properties was determined. The distribution of radioactivity in the tissue following injection of polysaccharide so tagged was also observed.

EXPERIMENTAL PROCEDURE

Preparation of Free Iodine From Radioactive Source

To several hundred microcuries of the sodium salt of carrier-free I^{131} was added 1 mg. of iodine as the sodium or potassium salt. Sodium nitrite (10–20 mg.) followed by 50 percent hydrochloric acid (several drops) was then added. The final volume was 5–10 ml. The liberated iodine was extracted with 2 ml. carbon tetrachloride.

Iodination With Free Iodine

To 1.0 ml. of a solution containing 2.5 mg. polysaccharide (preparation P-3R) was added 0.5 ml. of the CCl_4 solution of radioiodine (0.25 mg.). The mixture was shaken for a few minutes. Iodine was not completely extracted from the organic phase. The aqueous layer was made up to 5 ml., separated, and dialyzed through cellophane tubing (Visking) in running tap water for 48 hours. For radioactivity determination, 0.1 ml. in an aluminum cup was evaporated to dryness at 37° C. in the presence of a small amount of $NaHSO_3$. A standard was prepared by extracting 0.5 ml. of the CCl_4 solution of radioactive iodine (0.25 mg.) with a solution containing 10 mg. of Na_2CO_3. This solution was diluted to 10 times the final volume of the polysaccharide solution, and 0.1 ml. was evaporated to dryness in the presence of $NaHSO_3$. Radioactivity measurements showed that 3 percent of the iodine used was incorporated into the polysaccharide. On the basis of a molecular weight of 8 million, given by Kahler et al. (*4*), 190 atoms of iodine were attached to each molecule of polysaccharide. A portion of the iodopolysaccharide was assayed for tumor-necrotizing properties on sarcoma 37.

Iodination With Sodium Hypoiodite

To 1.0 ml. solution containing 2.5 mg. polysaccharide (P-3R) was added 0.1 ml. 10 percent Na_2CO_3 (10 mg.) followed by 0.5 ml. of the CCl_4 solution of radioiodine (0.25 mg.). The mixture was shaken immediately, with rapid decolorization of the organic phase. Following dilution to 5 ml., the aqueous phase was dialyzed for 72 hours in running tap water. A standard solution was prepared as described previously. Radioactivity measurements showed that 3.5 percent of the iodine was incorporated into the polysaccharide,

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[2] From the Kirstein Laboratory for Surgical Research, Beth Israel Hospital, Boston, and the Department of Surgery, Harvard Medical School, Boston, and the National Cancer Institute, Bethesda, Md.

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or 223 atoms of iodine per molecule of polysaccharide. A portion of this preparation was assayed for tumor-necrotizing properties on sarcoma 37. Table 1 gives the results of iodination with various quantities of iodine dissolved in the same volume of CCl_4. There is evidence of saturation of the polysaccharide within a wide range of iodine concentration.

TABLE 1.—*Iodination of polysaccharide*

Polysaccharide preparation	Iodine used	Iodine incorporated		Weight of Na_2CO_3 used for 2.5 mg. polysaccharide
		Atoms iodine per mole polysaccharide	Percent of iodine used	
	Milligrams			*Milligrams*
P-3R	1.25	226	0.72	10
	.25	223	3.54	
	.05	183	14.4	
P-8	1.0	390	1.5	20
	.2	268	5.3	
	.05	220	17.4	

ASSAY FOR TUMOR-NECROTIZING PROPERTIES

The assay method for tumor-damaging potency was similar to that described previously (*2*), and, in addition, eosin-hematoxylin-stained sections of paraffin-embedded tumor tissues were examined microscopically.[4] A mash of sarcoma 37 was implanted subcutaneously in CAF_1 mice. On the eighth day, the polysaccharide was injected intraperitoneally. The untreated polysaccharide was given in three dose levels; the two iodinated preparations were given in four dose levels. Other tumor-bearing mice in this experiment served as untreated controls. All injections were made in a constant volume of 0.5 ml. of a 0.9-percent solution of NaCl. The animals were all killed after 24 hours.

TABLE 2.—*Tumor-necrotizing effect of iodinated polysaccharide from* Serratia marcescens

Polysaccharide preparation	Iodination method	Dose	Number of mice used	Damage to tumor [1]	
				Gross positive/negative	Microscopic positive/negative
		Micrograms			
P-3R	None	5.0	[2] 4	2/0	2/0
		1.0	8	6/2	3/5
		.5	8	4/4	3/5
P-3R	Free iodine	10.0	[3] 4	3/0	3/0
		5.0	[3] 4	2/1	2/1
		2.0	4	3/1	3/1
		2.0	6	2/4	5/1
		1.0	[3] 8	3/4	0/7
P-3R	Sodium hypoiodite	10.0	4	4/0	4/0
		5.0	4	4/0	4/0
		2.0	4	4/0	4/0
		2.0	6	6/0	4/2
		1.0	[3] 8	7/0	6/1
Untreated controls			4	0/4	0/4
			8	0/8	0/8

[1] Damage to tumor with smaller doses was usually less extensive than with larger doses.
[2] 2 mice died within 24 hours.
[3] 1 mouse died within 24 hours.

[4] By Dr. Ross C. MacCardle, National Cancer Institute.

Low doses were selected so that the comparisons of potency would be made at dosages which were in the vicinity of minimal effect on the tumors (*2*). The data, summarized in table 2, indicate that the potency after treatment of polysaccharide with sodium hypoiodite was not diminished, while the treatment with free iodine resulted in some loss of potency.

RADIOACTIVITY MEASUREMENTS OF BLOOD AND TISSUE

Heparinized blood obtained from mice by decapitation, from rabbits by heart puncture, or plasma from humans by venipuncture was evaporated to dryness in aluminum cups of uniform size for 48 hours at 37° C. Standards were prepared by evaporating small aliquots of the injected material with the same quantity of blood or plasma in order to approximate the same dry weight and radioactivity of the samples. In the case of mice, 0.2 ml. of blood was used for each determination. Mouse tissues were weighed wet, and 200 mg. was used whenever possible. Since the dry weight of any given tissue obtained wet varied little from mouse to mouse, radioactivity analyses gave a fair degree of accuracy in comparing the changes of radioactivity of any given tissue with time. However, comparisons of different tissues with one another and with blood were subjected to the considerable error introduced by self-absorption of beta rays by the different dry weights of these tissues. For example, mouse liver, kidney, and tumor were 1.5, 1.9, and 1.4, respectively, times as heavy as blood after drying 200 mg. of each. In the data presented, correction for these variations in dry weight was not made. Radioactivity measurements were made with a Victoreen Geiger tube (bell type with end mica window weighing 4 mg./cm.^{2}). Counts were made on a Tracerlab autoscaler. All specimens were at least 5-10 times background.

Passage of Radioiodopolysaccharide Through a Bacterial Filter

McConnell (*9*) had found previously that polysaccharide was removed from concentrated solutions on passage through some bacterial filters. It was of interest to know more precisely how much polysaccharide, if any, was removed from dilute solutions.

Radioiodopolysaccharide (25 micrograms per milliliter) was filtered twice with suction through a small Mandler diatomaceous candle (fine porosity). Radioactivity measurement revealed 20-percent loss.

Rate of Production of Dialyzable Radioactive Material from Radioiodopolysaccharide at 37° C. by Freshly Homogenized Mouse Liver

The livers from six C57 black mice were homogenized and mixed with 90 ml. saline. To each of four tubes containing 20 ml. of the mixture was added 0.2 ml. containing 25 micrograms of radioiodopolysaccharide (P–8). One tube was immediately placed in boiling water for 5 minutes and stored at 4° C. The other tubes were incubated at 37° with occasional stirring for 1, 9, and 18 hours and were then placed in boiling water for 5 minutes. After centrifugation of the cold coagulated mixture, 10 ml. of supernatant was taken from each tube and dialyzed in running water for 3 days. Aliquots were evaporated to dryness for radioactivity analyses. The disappearance of radioactivity with time of incubation in two experiments is shown in table 3. The first tube was considered as zero time and assumed to have 100 percent of the radioactivity.

Table 3.—*Rate of production of dialyzable radioactive material from radioiodopolysaccharide by fresh-mouse-liver homogenate as shown by decrease in radioactivity after dialysis*

Experiment No.	Time	Radioactivity at zero time
	Hours	*Percent*
1	0	100
	1	78
	9	40
	18	29
2	0	100
	1	65
	7	62
	22	26

Distribution of Radioactivity in Tissue Following Injection of Radioiodopolysaccharide

Radioactive iodopolysaccharide was given to six normal mice, six tumor-bearing mice, a rabbit, and two patients. These were pilot experiments designed to give clues as to the rate of disappearance from the blood and suggestive evidence concerning distribution in tissues. Since radioactivity measurement of tissue gives information only on the distribution of the tagged atom (iodine), inference concerning movement of the entire polysaccharide molecule must be considered as provisional. In the event that the polysaccharide molecule may be broken down in the body into fragments, these radioactivity measurements may represent the distribution of those fragments which contain iodine rather than the distribution of the intact molecule. This is more likely to be the case late rather than soon after injection.

Normal Mice

Intravenous injection of 25 micrograms of iodopolysaccharide (P–8) in 0.5 ml. of saline was made into each of six C57 black mice. They were killed at fixed intervals by decapitation. On the basis of radioactivity injected and on the assumption of a blood volume of 10 percent of body weight, the radioactivity of 0.2 ml. of blood at zero time was calculated. The radioactivity of blood and tissues of each mouse was expressed as a percent of the activity of blood at zero time. These data are given in table 4.

Table 4.—*Radioactivity of unit wet weight of blood and organs of each of 6 normal mice expressed in percent of activity of blood at zero time at various intervals after injection of iodopolysaccharide*

Time (in hours)	Radioactivity of unit wet weight of—				
	Blood	Liver	Lung	Kidney	Thyroid
0.5	22	35	12	7	5
1	23	30	10	7	7
2	21	31	7	6	11
6	13	22	7	4	15
16	7	16	3	3	3
24	3	11	2	2	1.5

Tumor-Bearing Mice

Intravenous injection of 12.5 micrograms of iodopolysaccharide (P-8) in 0.5 ml. of saline was

made into each of 6 CAF_1 mice bearing 7-day-old sarcoma 37 in the muscles of one leg. Radioactivity measurements were made on tumor, blood, and liver of each mouse killed at various intervals. Gross and microscopic examination of the tumors revealed edema after 2 hours, and hemorrhage and edema after 5 hours. The results of radioactivity analyses are given in table 5.

TABLE 5.—*Radioactivity of unit wet weight of tumor, blood, and liver of each of 6 CAF_1 mice bearing sarcoma 37 expressed in percent of radioactivity of blood at zero time at various intervals after injection of iodopolysaccharide*

Time (in hours)	Radioactivity of unit wet weight of—		
	Tumor	Blood	Liver
0.5	3	24	82
1	6	26	74
2	5	64	62
5	5	15	43
14	7	32	46
26	3	7	34

NORMAL RABBIT BLOOD

A rabbit was given a 3-ml. intravenous injection containing 150 micrograms of radioiodopolysaccharide (P-8). Blood obtained by heart puncture at various intervals was used for radioactivity analyses. With a blood volume of 10 percent of body weight, the unit radioactivity of blood at zero time was calculated. The disappearance rate from the blood as percent of unit activity of blood at zero time is given in the following tabulation:

Time (in hours):	*Percent*
0.2	40
0.8	29
2.3	25
4.0	24
7.5	21

HUMAN BLOOD PLASMA [5]

A patient (R. G.) in the terminal stage of ovarian carcinomatosis receiving iodopolysaccharide (P-8) injections intravenously was given radioiodopolysaccharide (300 micrograms) as a fourth injection. The rate of disappearance of radioactivity from the blood was determined by frequent blood sampling. Urine was collected for 12-hour periods, and radioactivity measurements were made on evaporated aliquots. In the first 12-hour period following injection, the urine contained 2.3 percent of the radioactivity injected, and in the second 12-hour period 15 percent. No attempt was made to determine whether the urinary radioactive iodine was organically bound or ionic. A plasma volume of 5 percent body weight was assumed; the radioactivity of unit plasma at zero time was then calculated from a standard prepared with human plasma. The percent of the radioactivity at zero time exhibited by the specimens is shown in the next tabulation:

Time (in hours):	*Percent*
0.6	20
1.6	15
4.0	10
6.3	9.6

Another patient (L. K.) in the terminal stage of metastatic osteogenic sarcoma was given radioiodopolysaccharide (70 micrograms) intravenously as a second injection, and the plasma disappearance rate was determined. Urine analysis showed an output of 64 percent of the injected radioactivity in the first 12-hour period and 14 percent in the second 12-hour period. The radioactivity of plasma samples compared with that at zero time is given in the tabulation that follows:

Time (in hours):	*Percent*
0.2	66
0.5	19
1.0	15
2.2	13
3.4	9.3
4.7	8.8

DISCUSSION

Free iodine reacts by addition to many unsaturated substances, whereas sodium hypoiodite replaces active hydrogen by iodine in many substances, such as phenols, aromatic amines, aldehydes, and some ketones. It is interesting that approximately the same quantity of iodine was incorporated by each method of iodination. No precise formulation of the mode of attachment of iodine to the polysaccharide can be made because of lack of knowledge of much of its structure. The presence of protein contamination could be a serious disadvantage in these experiments because of the ease with which protein is tagged with alkaline radioactive iodine, as demonstrated by one of us previously (*11*). However, the polysaccharide was apparently freed of protein by trypsin digestion (*1*) as indicated by chemical

[5] Both cases are described in another publication (Cases 6 and 7 (*10*)).

and physicochemical criteria (*3*, *4*). Hartwell, Shear, and Adams (*3*) have shown that hydrolysis of the polysaccharide yields *d*-glucose, methyl pentose, *d*-glucosamine, and some phospholipid. Evidence of the phospholipid was obtained by finding glycerol, organically bound phosphorus, fatty acid, and nitrogen in excess of that accounted for by glucosamine. No determination was made of whether the fatty acid was unsaturated or not, other than that it was "solid." If unsaturation is present in this portion of the molecule, attachment of free iodine might possibly be explained. No aromatic nuclei have as yet been demonstrated in the polysaccharide, and the polysaccharide does not reduce Fehling's solution (*3*). This evidence for the absence of active hydrogen makes it difficult to understand the mode of reaction with sodium hypoiodite.

That the iodine linkage is a firm one is suggested by the experiments on production of dialyzable material from radioiodopolysaccharide by freshly homogenized mouse liver shown in table 3. After 18 hours 22 percent of the iodine was still nondialyzable. The iodine that was lost by dialysis may not have been ionic but may still have been organically bound. Further evidence for a firm bond may be deduced from the low iodine content of thyroid for 24 hours following injection of radioiodopolysaccharide in mice shown in table 4, and from the fact that radioactivity was found in the urine of mice and man as late as 24 hours after injection. Thyroid takes up only ionic iodine, but the urine might have contained iodine organically bound to fragments of the polysaccharide.

The rapid disappearance from the circulating blood to 20 percent of the radioactivity at zero time, with a prolonged maintenance at this low level, is in sharp contrast with the slow steady rate of disappearance from the blood of radioactive iodo-plasma protein demonstrated in dogs (*11*). Greatest radioactivity was found in liver, lung, and kidney, decreasing in this order. The high radioactivity noted in liver takes on added significance in view of the recent demonstration by one of us (*12*, *13*, *14*) of the importance of liver damage in the development of irreversibility in hemorrhagic shock in dogs and in view of the frequent occurrence of shock following the administration of polysaccharide to patients (*5*, *6*, *7*, *10*). The tumors examined contained surprisingly little radioactivity even though the necrotizing action occurred in these tumors.

CONCLUSIONS

The polysaccharide from *Serratia marcescens* was tagged with radioactive iodine without destruction of its tumor-necrotizing property by a procedure involving alkaline iodination.

Iodination of polysaccharide with varying amounts of iodine showed saturation of the molecule over a wide range of iodine concentration.

The rate of production of radioactive dialyzable material from radioiodopolysaccharide with fresh mouse liver homogenate was determined.

The rate of disappearance from the blood of intravenously injected radioiodopolysaccharide was measured in pilot experiments (mouse, rabbit, and man).

The distribution of radioactivity at various intervals after injection of radioiodopolysaccharide in the normal tissues and in tumor tissue of mice was measured in two experiments.

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CHEMICAL ALTERATION OF POLYSACCHARIDE FROM *SERRATIA MARCESCENS*. II. EFFECTS OF IODOPOLYSACCHARIDE IN PATIENTS WITH MALIGNANT TUMORS[1,2,3]

By THEODORE SACK and ARNOLD M. SELIGMAN

INTRODUCTION

The results of the administration of a tumor-necrotizing bacterial polysaccharide from *Serratia marcescens* to patients with cancer were reported by Brues and Shear (*1*), Holloman (*2*), and Oakey (*3*). Toxic reactions following the injection of this material into six patients with advanced malignant tumors were observed by one of us.[4] The major clinical effects consisted of fever and peripheral vascular collapse, together with numerous associated subjective and objective phenomena. The reactions were grossly similar to those seen following the intravenous administration of various pyrogenic materials to man (*4*).

The polysaccharide was approximately seven times as toxic in mice with tumors as in normal mice (*5*). Furthermore, hemorrhage was produced in both primary and transplanted tumors by less than a microgram of polysaccharide, although the degree of hemorrhage was greater with larger doses (*5*). In normal mice and mice with tumors, the polysaccharide produced a fall rather than a rise in temperature (*6*). This occurred in the presence of hemorrhage into tumor tissue, indicating that tumor destruction is not caused by the nonspecific pyrogenicity of the polysaccharide (*6*). In guinea pigs (*7*), rabbits (*8*), and dogs (*7*), the polysaccharide produced pyrogenic reactions and shock. The pyrogenic reaction in rabbits was particularly striking. Over a wide range of dosage, no correlation was found between the magnitude of the febrile response and the size of the dose (*8*). Of all species studied, mice appeared to be least susceptible to the polysaccharide. It has not been possible to administer doses of polysaccharide to man comparable with those that produce a minimum tumor-necrotizing effect in mice. The possibility of accomplishing this by graded increases in dose over a period of many days would appear unpromising because of the rapid development of tolerance to the material by man, animals (*5*), and tumors (*5*).

It is apparent, therefore, that the *S. marcescens* polysaccharide of Shear can be of therapeutic value in clinical cancer only if it is possible greatly to diminish its toxicity for man, thereby making it feasible to administer much larger doses than have previously been given.

To this end, the possibility of reducing the toxicity of polysaccharide by chemical means, without decreasing tumor-necrotizing properties, was explored. Radioactive iodine was used, and it was found that iodine could be attached to the polysaccharide without destroying its tumor-necrotizing effect (*9*). The rate of disappearance from the blood of polysaccharide so tagged and its distribution in the tissues, as indicated by radioactivity measurements, were determined in pilot experiments (*9*).

No significant decrease in the toxicity for man was obtained on iodination of the polysaccharide. The observations on the toxic reactions and the effect on tumors in seven patients form the basis of this report.

METHOD

Patients with inoperable or recurrent radioresistant malignant tumors were selected for the present study. In every case, the diagnosis was established previously by histologic examination of the tumor. Biopsy or autopsy material was obtained 2–7 days after administration of the iodopolysaccharide in four cases and after many months in two cases. In case 6, the effect on pulmonary metastases was determined by roentgenological examination only.

IODOPOLYSACCHARIDE ADMINISTRATION

Iodination of polysaccharide in the presence of sodium carbonate, as previously described (*9*), was performed with preparations P3R or P-8 (Shear). Intravenous administration was done either by a single injection, in small volume, over a period of 30–60 seconds, or by an infusion in

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[2] From the Kirstein Laboratory for Surgical Research, Beth Israel Hospital, Boston, and the Department of Surgery, Harvard Medical School, Boston.

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[4] SELIGMAN, A. M.: Unpublished observations.

TABLE 1.—*Protocol data for iodopolysaccharide injections*

Case No.	Record No.	Age	Sex	Pathologic diagnosis	Other diagnoses	Previous operations	Previous X-ray therapy	Organs known to be invaded by tumor	Estimated total tumor-tissue mass	Impaired vital capacity	Time of post injection biopsy or autopsy [1]
		Years									
1	90728 E. H	54	F	Renal-cell carcinoma.	Arterial hypertension; hypertensive cardiovascular disease with compensated cardiac hypertrophy; bronchial asthma.	Right nephrectomy (2 months earlier).	None	Right lung; right pleura.	2+	Yes	Autopsy (7.5 months).
2	91787 H. B	56	M	Gastric undifferentiated carcinoma.	Mild diabetes mellitus; history of botulism; cachexia.	Exploratory laparotomy (1 month earlier.)	None	Stomach, liver, pancreas, right adrenal, right kidney, gall bladder, lymph nodes, peritoneum and lung.	6+	Yes	Autopsy (2 days).
3	92049 V. E	38	F	Ovarian undifferentiated carcinoma.	History of severe post-radiation dermatitis, leukopenia, and gastrointestinal reaction; cachexia.	Left oophorectomy (10 months earlier); right oophorectomy and resection peritoneal masses (2 weeks earlier).	3,700 r to abdomen (ending 8 months earlier).	Parietal and visceral peritoneum.	4+	Yes	Biopsy (7 days; autopsy (14 days).
4	92717 S. D	67	F	Mammary scirrhous carcinoma.	Arterial hypertension; hypertensive cardiovascular disease with compensated cardiac hypertrophy; basal atelectasis.	Left radical mastectomy (5.3 years earlier).	9,600 r to left supraclavicular area (ending 12 months earlier).	Left chest wall and skin; left supraclavicular lymph nodes.	+	Yes	Biopsy (7 days).
5	92947 F. G	42	F	Mammary undifferentiated carcinoma.		Right radical mastectomy (10 months earlier).	12,000 r to right axilla, supraclavicular area, and mediastinum (ending 5.5 months earlier).	Spinal column, ribs, pelvis, femora.	3+	No	Autopsy (8 months).
6	93191 L. K	19	M	Osteogenic sarcoma (tibia).	Cachexia	Left mid-thigh amputation (17 months earlier).	None	Right lung; left pleura.	2+	Yes	
7	93611 R. G	33	F	Ovarian papillary adenocarcinoma	Malnutrition; severe anxiety.	Bilateral salpingo-oophorectomy (3 months earlier).	3,200 r to pelvis (ending 2 months earlier).	Pleura, mediastinum, peritoneum.	4+	Yes	Autopsy (5 days).

[1] Time after last polysaccharide injection.

TABLE 2.—*Additional protocol da*

Protocol data							Pyrogenic reaction							
							Primary rise		Temporary recovery		Secondary rise			Fin
Case No.	Injection No.	Day No.	Polysaccharide preparation	Dose	Intravenous administration [1]	Time for administration by drip	Maximum temperature	Time of peak [2]	Time of start of defervescence [2]	Lowest temperature reached	Time of start [2]	Maximum temperature	Time of peak [2]	Time star
				Micrograms		*Hours*	*°F.*	*Hours*	*Hours*	*°F.*	*Hours*	*°F.*	*Hours*	Hou
1	1	1	I-P3R	10	S. I.		104.0	3.0	5.0	100.2	8.0	101.6	10.0	10
	2	3	I-P3R	20	S. I.		102.2	3.0	5.0	99.2	6.0	102.0	8.0	10
	3	4	I-P3R	40	S. I.		102.0	2.5	3.0	100.2	4.0	101.2	5.5	6
	4	6	I-P3R	80	S. I.		102.8	1.5	2.0	100.0	5.0	102.8	6.5	7
	5	70	I-P3R	10	S. I.		102.0	3.5	5.5	99.0	7.0	102.0	9.5	10
	6	73	I-P3R	20	S. I.		102.0	2.5	4.5					4
	7	74	I-P3R	40	S. I.		102.2	2.5	5.5					5
	8	75	I-P3R	60	S. I.		101.2	2.0	4.0					3
	9	77	I-P3R	100	S. I.		103.0	3.0	8.0					8
2	1	1	I-P3R	10	S. I.		104.6	6.0	7.5					9
3	1	1	I-P3R	5	S. I.		105.0	4.0	5.5	99.2	9.0	104.0	12.0	14
	2	6	I-P3R	10	S. I.		103.2	3.5	5.0					5
	3	8	I-P3R	20	S. I.		103.0	4.0	4.5					4
4	1	1	I-P3R	2	S. I.		102.0	7.0	7.5					7
	2	2	I-P3R	20	S. I.		105.4	3.5	4.0	102.0	6.5	102.4	7.5	10
	3	4	I-P3R	20	S. I.		101.4	2.5	4.0	99.8	5.0	101.2	6.0	6
	4	6	I-P3R	40	S. I.		103.4	3.0	4.5					4
5	1	1	I-P3R	20	Drip	4.0	104.0	3.0	5.0					5
	2	5	I-P3R	40	do	7.0	104.0	4.5	8.0					8
	3	7	P8	10	do	2.5	102.0	2.0	2.5	98.6	7.5	99.2	8.5	8
	4	9	I-P8	50	S. I.		103.0	3.0	3.5	100.0	6.0	101.0	9.0	9
6	1	1	I-P8	10	Drip	5.0	105.0	2.0	2.5	100.4	3.5	104.2	6.5	7
	2	3	I-P8	20	do	4.5	104.0	4.5	4.5	100.4	6.0	103.0	7.0	8
	3	7	I-P8	80	do	6.5	102.0	1.5	1.5	100.0	2.5	103.0	3.5	3
	4	10	I^{*}-P8 [3]	70	S. I.		102.0	3.5	3.5					3
	5	14	I-P8	160	Drip	4.5	104.6	4.0	5.0	99.0	6.5	102.0	8.0	8
7	1	1	I-P8	5	do	2.0	104.2	3.5						4
	2	5	I-P8	20	do	4.5	105.0	4.0						5
	3	8	I-P8	80	do	7.0	105.0	6.5						7
	4	30	I^{*}-P8 [3]	300	S. I.		104.6	4.5	5.5	98.6	9.0	102.0	20.0	2
	5	33	P8	3,000	S. I.		103.6	4.0	4.5	101.6	7.0	102.0	10.0	2

[1] Single injection (S. I.) or continuous drip.
[2] Time refers to number of hours after polysaccharide injection or after the start of a continuous drip of polysaccharide.
[3] I^{*}, Radioactive iodopolysaccharide. (See reference (*9*).)

nal protocol data and summary of certain clinical changes following iodopolysaccharide injection

ction					Blood-pressure changes (systolic)									
ary rise		Final defervescence		Total duration of fever above 100°	Initial blood pressure	Primary blood-pressure fall			Temporary recovery		Secondary blood-pressure fall			Time start sustained recovery [2]
xi-mum-ra-re	Time of peak [2]	Time of start [2]	Time when afebrile [2]			Time of start of fall [2]	Minimum blood pressure	Time of reaching minimum [2]	Time of start [2]	Maximum blood pressure	Time of start [2]	Minimum blood pressure	Time of reaching minimum	
F.	*Hours*	*Hours*	*Hours*	*Hours*	*Mm. Hg*	*Hours*	*Mm. Hg*	*Hours*	*Hours*	*Mm. Hg*	*Hours*	*Mm. Hg*	*Hours*	*Hours*
1.6	10.0	10.5	27.0	10.0	160	3.0	100	8.0	8.5	140	14.0	90	15.5	35.
2.0	8.0	10.5	15.0	10.0	140	2.5	120	6.0	6.5	140	11.0	110	20.0	23.
1.2	5.5	6.5	8.0	7.0	128	3.0	120	3.5	4.0	152	9.0	100	19.0	19.
2.8	6.5	7.0	11.0	10.0	190	8.0	92	17.0						21.
2.0	9.5	10.0	12.0	8.0	172	3.5	70	16.5	17.0	98	19.0	62	21.0	21.
		4.5	11.0	8.0	138	3.5	90	10.0						11.
		5.5	10.0	8.0	130	3.5	110	8.5						9.
		3.5	9.0	6.0	158	1.5	90	4.5	7.5	122	12.5	92	15.0	15.
		8.0	13.0	10.0	164	3.0	120	11.5						18.
		9.5	20.0	15.0	140	3.5	84	6.0						11.
4.0	12.0	14.5	28.0	20.0	110	4.0	68	7.5	16.0	100	20.0	76	30.0	30.
		5.0	20.0	13.0	110	9.0	90	12.5						15.
		4.5	12.0	7.0	108	3.5	104	6.0	6.5	120	10.0	84	12.5	14.
		7.5	16.0	12.0	160	2.5	90	8.0						11.
2.4	7.5	10.5	20.0	17.0	120	3.0	88	8.0						9.
1.2	6.0	6.5	12.0	8.0	128	2.0	102	4.5						8.
		4.0	9.0	5.0	140	3.0	118	3.5						9.
		5.0	20.0	13.0	130	2.5	72	9.0						23.
		8.0	12.0	8.0	130	5.0	92	12.0						12.
9.2	8.5	8.5	9.0	3.0	140	2.5	110	7.0						12.
1.0	9.0	9.5	9.5	6.0	140	3.0	102	6.5						12.
4.2	6.5	7.0	23.0	13.0	120	2.5	100	6.5						9.
3.0	7.0	8.0	12.0	8.0	120	4.5	88	8.0						8.
3.0	3.5	7.5	10.5	8.0	120	6.5	100	12.0						12.
		3.5	4.0	1.0	130	1.5	100	8.0						10.
2.0	8.0	8.5	10.5	5.0	120	4.0	100	13.0						13.
		4.0	18.0	13.0	110	2.0	82	4.5						9.
		4.5	16.0	12.0	100	3.0	80	7.5						8.
		7.0	16.0	12.0	110	3.0	70	6.0						10.
2.0	20.0	22.0	24.0	17.0	90	2.0	10	4.5						12.
2.0	10.0	22.5	25.0	23.0	100	1.0	40	2.0	3.5	62	8.0	38	22.0	23.

ABLE 2.—*Additional protocol data and summary of certain clinical changes following iodopolysaccharide injection*

Pyrogenic reaction								Blood-pressure changes (systolic)											Pulse rate changes				Respiratory rate changes			
porary overy		Secondary rise			Final defervescence		Total duration of fever above 100°	Initial blood pressure	Primary blood-pressure fall			Temporary recovery		Secondary blood-pressure fall			Time of start of sustained recovery [2]	Additional evidence of shock	24 hours after injection		Initial rate per minute	Maximum rate per minute	Time of reaching maximum rate [2]	Time of returning to initial or usual level [2]	Initial rate per minute	Maximum rate per minute	Time of reaching maximum rate [2]	Time of returning to initial or usual level [2]
	Lowest temperature reached	Time of start [2]	Maximum temperature	Time of peak [2]	Time of start [2]	Time when afebrile [2]			Time of start of fall [2]	Minimum blood pressure	Time of reaching minimum [2]	Time of start [2]	Maximum blood pressure	Time of start [2]	Minimum blood pressure	Time of reaching minimum			Average blood pressure	Deviation from initial blood pressure							
	°F.	*Hours*	*°F.*	*Hours*	*Hours*	*Hours*	*Hours*	*Mm. Hg*	*Hours*	*Mm. Hg*	*Hours*	*Hours*	*Mm. Hg*	*Hours*	*Mm. Hg*	*Hours*	*Hours*		*Mm. Hg*	*Mm. Hg*			*Hours*	*Hours*			*Hours*	*Hours*
	100.2	8.0	101.6	10.0	10.5	27.0	10.0	160	3.0	100	8.0	8.5	140	14.0	90	15.5	35.0	0	92	−68	74	124	3.0	33.0	28	36	2.5	28.0
	99.2	6.0	102.0	8.0	10.5	15.0	10.0	140	2.5	120	6.0	6.5	140	11.0	110	20.0	23.0	0	120	−20	120	140	2.0	19.0	24	26	1.0	19.0
	100.2	4.0	101.2	5.5	6.5	8.0	7.0	128	3.0	120	3.5	4.0	152	9.0	100	19.0	19.5	0	134	+6	90	130	.5	15.0	24	32	9.0	15.0
	100.0	5.0	102.8	6.5	7.0	11.0	10.0	190	8.0	92	17.0						21.0	0	130	−60	118	130	1.5	17.0	26	32	5.0	36.0
	99.0	7.0	102.0	9.5	10.0	12.0	8.0	172	3.5	70	16.5	17.0	98	19.0	62	21.0	21.5	0	100	−72	92	138	2.5	49.0	20	24	1.0	27.0
					4.5	11.0	8.0	138	3.5	90	10.0						11.0	0	130	−8	88	122	1.5	14.5	22	28	1.0	4.0
					5.5	10.0	8.0	130	3.5	110	8.5						9.0	0	156	+26	88	140	.5	11.0	20	28	2.0	14.0
					3.5	9.0	6.0	158	1.5	90	4.5	7.5	122	12.5	92	15.0	15.5	0	150	−8	100	138	1.5	12.0	22	22		
					8.0	13.0	10.0	164	3.0	120	11.5						18.0	0	160	−4	100	158	2.5	15.0	20	28	8.0	10.0
					9.5	20.0	15.0	140	3.5	84	6.0						11.0	+	102	−38	80	140	3.5	24.0	30	48	2.5	17.0
	99.2	9.0	104.0	12.0	14.5	28.0	20.0	110	4.0	68	7.5	16.0	100	29.0	76	30.0	30.5	++	90	−20	88	152	5.0	33.5	24	30	2.5	30.0
					5.0	20.0	13.0	110	9.0	90	12.5						15.0	0	110	0	80	152	3.5	19.0	15	26	2.0	20.5
					4.5	12.0	7.0	108	3.5	104	6.0	6.5	120	10.0	84	12.5	14.0	0	100	−8	88	130	2.5	13.0	24	28	3.0	10.0
					7.5	16.0	12.0	160	2.5	90	8.0						11.5	0	110	−40	94	120	3.5	14.0	20	36	3.5	13.0
	102.0	6.5	102.4	7.5	10.5	20.0	17.0	120	3.0	88	8.0						9.0	0	104	−16	80	148	3.0	20.0	20	40	.5	19.0
	99.8	5.0	101.2	6.0	6.5	12.0	8.0	128	2.0	102	4.5						8.0	0	140	+12	100	130	1.5	12.0	24	40	5.5	12.0
					4.0	9.0	5.0	140	3.0	118	8.5						9.0	0	160	+20	100	138	1.0	11.0	28	40	3.5	11.0
					5.0	20.0	13.0	130	2.5	72	9.0						23.0	+	90	−40	100	140	1.5	20.0	20	48	4.5	20.0
					8.0	12.0	8.0	130	5.0	92	12.0						12.5	0	110	−20	110	138	4.0	14.0	24	38	3.5	18.0
	98.6	7.5	99.2	8.5	8.5	9.0	3.0	140	2.5	110	7.0						12.0	0	120	−20	82	110	2.5	10.5	20	34	2.0	13.0
	100.0	6.0	101.0	9.0	9.5	9.5	6.0	140	3.0	102	6.5						12.0	0	130	−10	84	120	2.5	10.5	20	48	1.0	16.0
	100.4	3.5	104.2	6.5	7.0	23.0	13.0	120	2.5	100	6.5						9.0	0	120	−10	92	164	1.5	25.0	20	42	.5	19.0
	100.4	6.0	103.0	7.0	8.0	12.0	8.0	120	4.5	88	8.0						8.5	0	110	−10	100	140	6.5	20.0	24	48	1.0	14.0
	100.0	2.5	103.0	3.5	7.5	10.5	8.0	120	6.5	100	12.0						12.5	0	110	−10	100	150	3.5	11.0	20	34	1.5	15.0
					3.5	4.0	1.0	130	1.5	100	8.0						10.0	0	120	−10	100	140	1.5	18.0	20	32	3.5	8.0
	99.0	6.5	102.0	8.0	8.5	10.5	5.0	120	4.0	100	13.0						13.5	0	114	−6	90	150	3.0	14.0	20	44	4.0	9.5
					4.0	18.0	13.0	110	2.0	82	4.5						9.0	0	100	−10	90	140	3.5	18.0	26	32	2.5	4.5
					4.5	16.0	12.0	100	3.0	80	7.5						8.5	0	100	0	98	130	2.0	18.0	20	36	4.5	16.0
					7.0	16.0	12.0	110	3.0	70	6.0						10.0	+	90	−20	90	160	7.5	23.0	20	24	2.5	7.0
5	98.6	9.0	102.0	20.0	22.0	24.0	17.0	90	2.0	10	4.5						12.0	++	80	−10	100	180	2.0	36.0	20	22	12.0	22.0
5	101.6	7.0	102.0	10.0	22.5	25.0	23.0	100	1.0	40	2.0	3.5	62	8.0	38	22.0	23.0	++	60	−40	112	180	2.0	48.0	14	16		

drip of polysaccharide.

1–2 l. of 5-percent glucose in water or saline over periods of 2–7 hours. Intervals between injections varied from 1 to 64 days.

Clinical Observations

Patients were hospitalized for the period of polysaccharide administration. A special-duty nurse was in constant attendance on the day of injection. Temperature, pulse, respiration, and blood-pressure recordings were made at half-hour intervals during the first 12 hours after injection. When indicated, these measurements were made at 15-minute intervals. The intervals between these clinical measurements were increased when some degree of stability of the cardiovascular dynamics was attained. Oral temperatures were taken except during shaking chills and shock or depression from heavy sedation. At such times rectal temperatures were taken. All temperatures given herein are oral and were obtained, in some instances, by subtraction of 1° F. from the rectal temperatures.

Blood-cell counts, urinalyses, and blood nonprotein nitrogen determinations were made at intervals during the period of hospitalization.

The appearance of pulmonary and bone metastases was followed by means of roentgenograms.

Supportive Treatment

Patients were maintained on as nourishing a diet as they could tolerate during their hospital stay and in some instances were given supplementary therapy in the form of intravenous glucose, plasma, or blood when indicated.

Sedatives were administered before most of the polysaccharide injections to lessen apprehension and other unpleasant symptoms, such as pain, severe dyspnea, and malaise. Dilaudid (2–4 mg.) or morphine sulfate (8–15 mg.), subcutaneously, occasionally supplemented with oral barbiturates, proved effective. Oxygen was administered during periods of severe dyspnea, cyanosis, and shock. Intravenous fluid was given to make up for water loss from sweating and to meet the day's requirement. Neosynephrine and surgical pituitrin were administered when the systolic blood pressure dropped below 80 mm. Hg but were withheld in a few instances in order to follow the course of the reaction without complicating influences. Pressor drugs were given either in intermittent intramuscular injections or in highly diluted form intravenously. Plasma and whole-blood tranfusions were administered when there was other clinical evidence of shock.

RESULTS

The case reports for seven patients given iodopolysaccharide follow. Representative curves showing the course of the temperature, pulse rate, respiratory rate, and blood-pressure changes are given in figures 1–4. Tables 1 and 2 give the protocol data, and table 2 summarizes these changes for all the post-injection reactions.

Case 1

Diagnosis: Hypernephroma with pulmonary metastases.—This 53-year-old woman developed symptoms and signs referable to the right side of her chest 10 months before admission. Five months later right-sided pleural effusion was demonstrated. Three months later she developed hemoptysis, a mass in the right flank was discovered, and right-sided pulmonary metastases were demonstrated. Nephrectomy was performed for hypernephroma with invasion of veins; 2 months after operation polysaccharide was given.

She had a past history of mild bronchial asthma and moderate hypertension of at least 6 years' duration.

On admission, she was ambulatory and relatively well nourished. She had an occasional unproductive cough, exertional dyspnea, and transient, mild twinges of pain

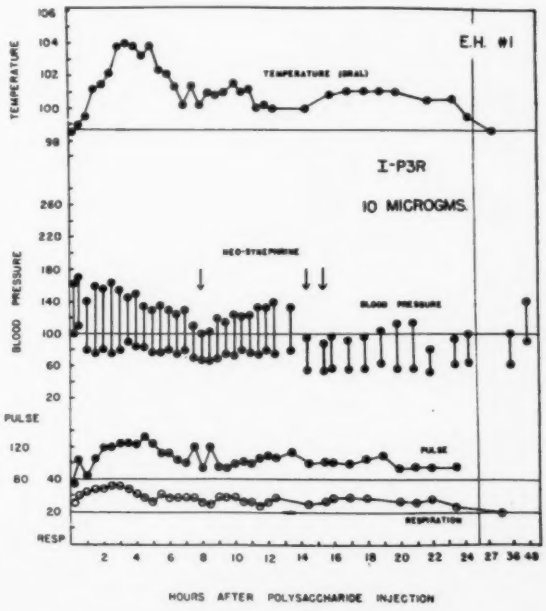

FIGURE 1.—Case 1.

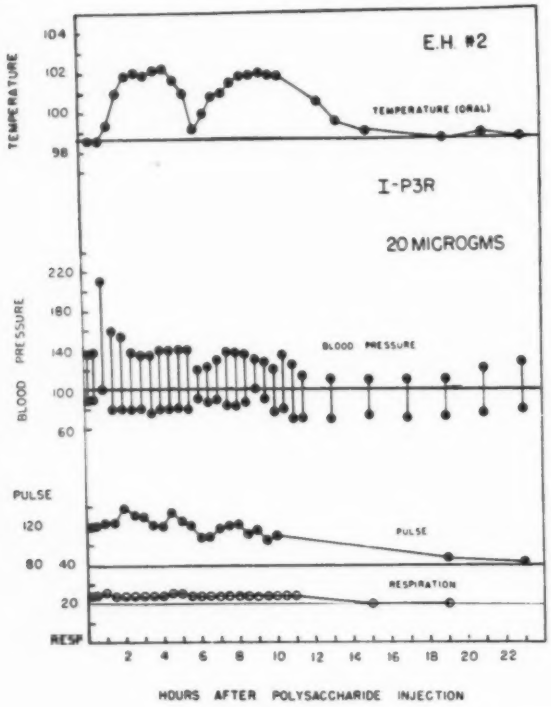

FIGURE 2.—Case 1.

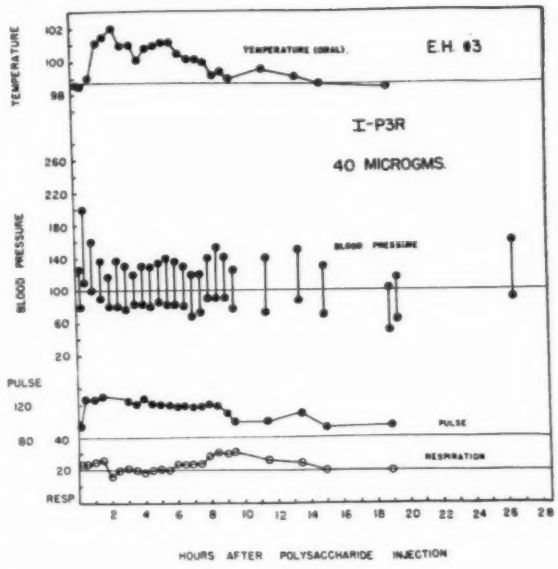

FIGURE 3.—Case 1.

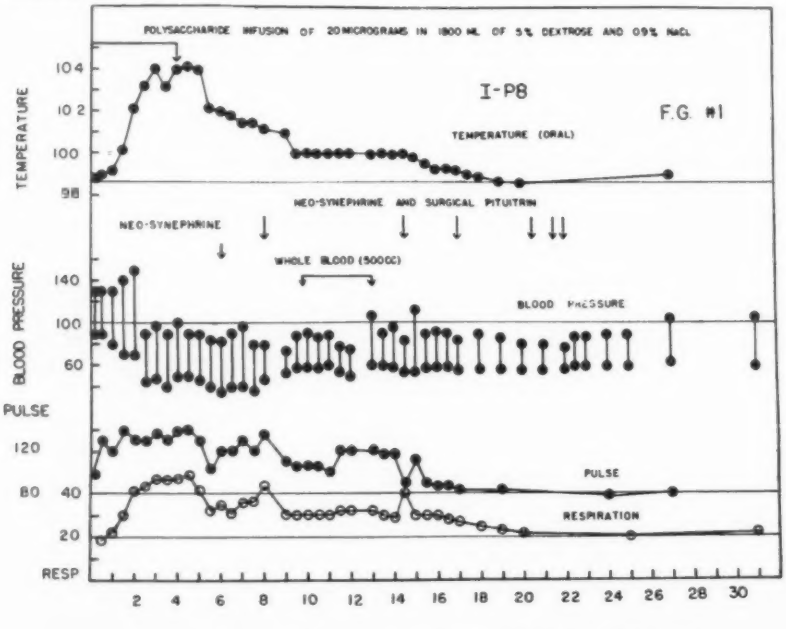

FIGURE 4.—Case 5.

in the right side of her chest. Her temperature was normal, pulse 90 per minute, respirations 20 per minute, and blood pressure 175/90 mm. Hg. She had moderate arteriosclerotic retinopathy, right pleural effusion, and fine rales in the right upper part of her chest. Her heart was greatly enlarged, and there was a long, rough, systolic murmur at the base, and a soft, systolic murmur at the apex. The urine was normal, the erythrocyte count was 4 million, with 76 percent hemoglobin, and the leukocyte count was 6,900.

By roentgenogram, the right lung showed pleural effusion and more metastatic implants than previously. The left lung was normal.

Clinical course.—Between the seventh and twelfth hospital days, inclusive, the patient was given four injections of iodopolysaccharide according to the schedule given in table 2. Each injection was followed by a severe pyrogenic reaction, accompanied by peripheral vascular collapse. The reaction following the first injection was the most severe of the four. It was ushered by a severe, shaking chill, accompanied by dyspnea, pallid cyanosis, severe lumbar backache, abdominal cramps, nausea, and vomiting. The dyspnea continued during the period of elevated temperature; the cyanosis, nausea, and vomiting lasted until the ninth post-injection hour. After the first 8 hours, marked malaise, fatigue, and weakness constituted the major discomfort. Figure 1 shows the course of changes in temperature, pulse rate, respiratory rate, and blood pressure during this reaction. Figures 2 and 3 show the changes during the next two reactions, which were less severe. The chills were not so marked, the dyspnea, cyanosis, and gastrointestinal symptoms were less severe, and the myalgia was slight. There was moderate asthmatic dyspnea from 10 to 15 hours after the fourth injection.

From the first day of iodopolysaccharide administration until the day of discharge, the major clinical change in the patient consisted of progressive and severe weakness, as well as increase in the exertional dyspnea. There was no definite evidence of cardiac decompensation. She developed severe herpes simplex of the lips and oral mucosa about 36 hours after the first injection; this progressed during subsequent treatments, and became extensive and painful.

Although relative hypotension tended to persist long after other evidence of acute toxicity had disappeared, her blood pressure had gradually risen to its previous hypertensive level by the time of discharge from the hospital.

There was never any significant suppression of urine formation. There was albuminuria on the day after the third injection but none thereafter. The erythrocyte counts and hemoglobin levels showed no essential change from their initial values. However, the leukocyte count was 13,400 on the day after the third injection, 24,200 on the day after the fourth injection, and 5,600 four days after the fourth injection. Roentgenograms of the chest, taken on the eleventh and sixteenth hospital days, showed a moderate increase in pleural effusion but no other notable changes.

The patient was discharged on the sixteenth hospital day, feeling considerably more weakened and fatigued than she had before the administration of iodopolysaccharide had begun. During the subsequent 2 months the patient remained at home. She was seen at approximately 2-week intervals, and additional roentgenograms of the chest showed progression of the metastases and effusion. Her course during these 2 months consisted of slow, progressive recovery from the debilitating effect of the polysaccharide injections. During this period at home she suffered right-sided chest pain of varying intensity, hacking cough, and occasional hemoptysis. Her appetite became somewhat improved during the second month, she felt a little stronger and maintained her body weight. There was slow and steady increase in her exertional dyspnea, but there was no evidence of cardiac decompensation. Her blood pressure remained around 170/90 mm. Hg.

Second hospital admission.—She was re-admitted 2 months after her previous discharge, for a second course of iodopolysaccharide injection. She was paler and somewhat less well nourished. She showed a little more air hunger on slight exertion. Her chest showed essentially the same physical signs as it had 2 months earlier, and there were no other significant physical changes. Her temperature was 98°, her pulse rate 90 per minute, and her respiratory rate 24 per minute. Her blood pressure was 168/90 mm. Hg.

Her urine had a specific gravity of 1.020 and was free of albumin. The erythrocyte count was 4.38 million, hemoglobin 12.3 gm., leukocyte count 9,300, and her blood nonprotein nitrogen 42 mg. percent.

Clinical course.—The patient was given five intravenous injections of iodopolysaccharide according to the schedule indicated in table 2.

In anticipation of some loss of resistance to the toxic action of the iodopolysaccharide in the 2-month interval, the patient was again started with a small dose, and subsequent doses were increased. Dilaudid was given before each injection. All the post-injection reactions followed the same pattern as did those in the first series. The reaction after the first injection of this series was of appreciably greater severity than that following the fourth injection of the earlier series. There were chilly sensations, cyanosis, dyspnea, myalgia, nausea, and vomiting of moderate severity. The temperature reached a maximum of 102°, but there was no fever after the thirteenth hour. The blood pressure fell to a minimum of 62 mm. Hg, systolic. It still had not risen above 140, systolic, 72 hours after the injection. The characteristic general weakness was marked. Moderate herpes appeared in 30 hours.

Injection 6 was followed by a reaction of slightly greater severity than was suffered after the previous one. The chill and muscle pain were much more severe, in spite of preliminary administration of dilaudid, which had to be repeated within the first 2 hours. The cyanosis and dyspnea were moderate. There was no nausea or vomiting, and the major symptom after the first 4 hours was severe generalized weakness.

The reactions after the patient's subsequent three in-

jections were only a little less severe than the previous ones in their early, acute phases. The extent and duration of the changes in temperature, blood pressure, pulse, and respiratory rates were, however, notably less marked. (See table 2.) On the other hand, the debilitating effect of each reaction was of great severity. There was little or no recovery from this weakness between injections, and this symptom, consequently, became more and more prominent. After the ninth iodopolysaccharide injection, her strength was so sapped that further administration of the material was considered inadvisable.

Urinalysis 3 days after the last polysaccharide injection showed no abnormality. Erythrocyte count on the same day was 4.3 million, hemoglobin 11.7 gm., and leukocyte count 21,000.

She was discharged on the fourteenth hospital day, looking considerably sicker than she had on admission. Physical examination at the time of discharge revealed a normal temperature, a pulse rate of 86 per minute, a respiratory rate of 22 per minute, and a blood pressure of 160/90 mm. Hg. There was evidence of some increased fluid in the right pleural space. There were no other notable physical changes.

During the subsequent 8 weeks, there was slight recovery of strength, notwithstanding the fact that exertional dyspnea was greater. Her cough was more persistent, and hemoptysis was more frequent. She was seen several times during this period in the outpatient clinic. There was no evidence of cardiac decompensation. Roentgenograms of the chest showed progressive increase in the amount of pleural fluid and parenchymal density on the right side.

During the subsequent 5 months, she deteriorated steadily, with progressive loss of strength and increase in dyspnea, chest pain on the right side, cough, hemoptysis, and loss of weight. During the last 2 months of this period, she was confined to bed almost completely and required frequent doses of demerol to control her chest pain.

Third hospital admission.—She was admitted for the last time exactly 7 months after her previous discharge. This time she was in a terminal state, showing marked dyspnea, malnourishment, and pallor. She had a frequent cough, hemoptysis, and severe pain in the right side of her chest, which showed no respiratory motion at all. There were several soft nodules in the ribs of that side. Laboratory examination showed slight albuminuria, an erythrocyte count of 2.5 million, and a nonprotein nitrogen of 40 mg. percent. Roentgenogram of the chest showed obliteration of the entire right-lung field with opaque shadow and destruction of the costal bone. The left-lung field was clear. The patient could tolerate very little food or fluid by mouth. On the fifth hospital day, she became more dyspneic, and her blood pressure fell from 160/80 to 90/50 mm. Hg. She died 3 days later, 7.5 months after her last polysaccharide injection.

Post-mortem examination.—There were metastatic lesions in the left kidney and adrenal. The mediastinum, right lung, right pleural space, and chest wall were almost completely replaced by metastatic tumor, with destructive involvement of most of the costal bone on the right; there were multiple small metastatic nodules in the left lung. Small areas of necrosis were seen in the central portions of the tumor masses. This condition was not related to polysaccharide injections given 7.5 months before.

Case 2

Diagnosis: Undifferentiated carcinoma of the stomach with extension to the duodenum, biliary tract, pancreas, and colon.—This 56-year-old man had a 5-year history of mild diabetes mellitus, a past history of penumonia, appendectomy, and botulism. Six months before admission to the hospital, he began to develop weakness, dyspnea, postprandial nausea, and sensation of an epigastric lump. These symptoms progressed; he lost weight. Four months after the onset of his illness, he had some hematemesis, and icterus was first noted. Exploratory laparotomy was performed at another hospital 1 month later, when he was found to have an inoperable carcinoma of the stomach, involving the duodenum, biliary tract, pancreas, and colon.

During the following 4 weeks at home, his symptoms progressed rapidly, he vomited nearly everything he took by mouth, had hematemesis on two occasions, and became more emaciated and jaundiced.

On admission, he appeared well-developed, emotionally tense, moderately cachectic, and complained of severe nausea. He was very pale and deeply icteric. His temperature was 99, pulse rate 90 per minute, respirations 20 per minute, and blood pressure 134/78 mm. Hg. The chest was clear, except for considerable elevation of both diaphragms; the heart was not enlarged; and the abdomen was moderately distended. There was a right lower quadrant appendectomy scar, a right upper quadrant vertical laparotomy scar, an ovoid, firm, moderately tender mass in the right upper quadrant almost as large as a football. There were several small, firm, inguinal lymph nodes.

His urine was normal except for the presence of a large amount of bile. The erythrocyte count was 3 million, with 67 percent hemoglobin, the leukocyte count 9,900 with 87 percent polymorphonuclear forms, the blood sugar 133 mg. percent, the blood nonprotein nitrogen 47 mg. percent, the icterus index 88, and the total serum protein 4.52 gm. percent.

Clinical course.—During the first 6 days in the hospital, the patient was hydrated by parenteral means, the diabetes was controlled with insulin, and he was given 2,500 cc. of whole blood and an infusion of 45 gm. of amino acids. His hemoglobin rose to 82 percent. Although he took nothing by mouth, abdominal distension, nausea, regurgitation, and vomiting continued. He also had a hacking cough and several attacks of dyspnea. During this period, he developed minimal pitting edema of his lower extremities and signs of fluid in his abdomen.

On the morning of the seventh hospital day, an intravenous infusion of 1,500 cc. of 10 percent dextrose in 0.9 percent saline (containing 25 units of regular insulin) was started. Soon after this, 10 micrograms of iodopolysaccharide was injected intravenously. A half hour later

he suffered a severe shaking chill, marked dyspnea, moderate cyanosis, and abdominal cramps. The chill lasted 30 minutes; the dyspnea continued for about 15 hours; he vomited repeatedly. From the fourth to the tenth hour there were long periods of Cheyne-Stokes respiration. In the tenth post-injection hour, respiratory distress was greatly relieved by gastric intubation and the removal of 700 cc. of fluid and a considerable quantity of gas. Intubation was continued.

His 24-hour clinical chart showed the following characteristics: The temperature rose steadily and reached its maximum level of 104.6° 6 hours after injection. It remained above 104° F. for 4 hours, then began to decline steadily, and reached normal 11 hours later. During the chill, the blood pressure rose from an initial level of 140/80 to 160 mm. Hg systolic, but it dropped to 90/50 mm. Hg 2 hours later. Following the injection of neosynephrine, the blood pressure rose to 120, systolic, and remained there for a brief period, and then declined to 84/60 4 hours later. From that time until the eleventh post-injection hour, the blood pressure fluctuated between 84 and 96 systolic and 60 and 70 diastolic, with transient minor rises following small intramuscular doses of neosynephrine and pituitrin. Shock was manifest by clammy skin and depressed sensorium. The injection of 500 cc. of plasma resulted in an elevation of the blood pressure to 104 systolic; it fluctuated around 100/60 for the next 24 hours.

Twenty-four hours after the polysaccharide injection, the patient looked and felt extremely weak but was otherwise relatively comfortable. His temperature was normal, blood pressure about 100/60, and pulse and respiratory rates were at satisfactory levels. This general state continued until 31 hours after polysaccharide injection. At this time, during an intravenous infusion containing 45 gm. of parenteral amino acids, he suffered a severe shaking chill, his temperature rose to 103.2°, his blood pressure dropped to 60/40, and he became cold and clammy. He was given 500 cc. of whole blood and repeated injections of neosynephrine and pituitrin. He showed a good response until about 10 hours after the chill, when his blood pressure began to decline and failed to respond to pressor drugs. He went into profound shock and died 15 hours after the second chill and 46 hours after polysaccharide injection.

Post-mortem examination.—There was ulcerated, obstructing carcinoma of the pylorus, with metastases to the regional, mesenteric, and peri-aortic lymph nodes, liver, peritoneum, right adrenal, right kidney, left lung, and gall bladder, with extension to the pancreas, first and second portions of the duodenum, the ampulla of Vater, and the stomach. Hemorrhage or necrosis was seen in the center of large tumor masses and could not be attributed to the administration of polysaccharide. Microscopic examination of various parts of the tumor failed to show either early or extensive necrosis.

Case 3

Diagnosis: Metastatic undifferentiated carcinoma of the ovary.—This 38-year-old woman developed symptoms referable to the pelvic region a year earlier. Three months later a large pelvic mass was found, which proved to be a tumor of the left ovary with dense adhesions to surrounding structures. The main tumor mass was resected. Pathologic examination showed it to be a malignant teratoma. For about 1 month post-operatively, she received a total of 3,700 r of X-radiation to her pelvis. At the end of this period, she developed a severe reaction which included marked gastrointestinal symptoms, dermatitis, fever, and leukopenia. She was desperately ill for 2 weeks, and then her symptoms subsided. She remained well until 1 month before admission when she developed progressive low abdominal and rectal pain and anorexia.

Physical examination on admission showed a pale, slightly cachectic woman. Her temperature was 98.6, pulse rate 80 per minute, respiratory rate 18 per minute, and blood pressure 110/70 mm. Hg. Her heart and lungs were normal. There was a large abdominal mass rising out of the pelvis, some pigmentation of the skin of the lower abdomen, and palpable masses on vaginal and rectal examination. Her urine at admission was normal; erythrocyte count was 4.65 million, hemoglobin 12.1 gm., leukocyte count 5,600 with 61 percent polymorphonuclear forms, 32 percent lymphocytes, 6 percent monocytes, and 1 percent eosinophils.

Because the right ovary had not been removed at the previous operation, it was considered advisable to determine by operation whether the mass was confined to that organ. A football-size tumor immediately beneath the abdominal wall was found, which originated from the right adnexa. There were innumerable implants up to 3 inches in diameter on the peritoneal surface of the large intestine and mesentery. Some of these were pedunculated. The pelvis was choked with a firm, lobulated mass of tumor tissue which encircled and was adherent to the rectosigmoid junction. Bloody fluid was present in the peritoneal cavity. Because of anticipated polysaccharide injections, it was deemed advisable to remove as much of the bulk of tumor tissue as possible. Therefore the football-size mass was removed, the largest pedunculated masses were resected from the small intestine, transverse colon, and rectosigmoid junction, and most of the tumor was removed from the cul-de-sac. Much of the tumor contained areas of hemorrhage and necrosis. Microscopically, it was undifferentiated carcinoma. Postoperatively, the patient had abdominal pain and recurrent nausea and vomiting. Two weeks later, following a transfusion of 500 cc. of whole blood, her erythrocyte count was 3.8 million with 11.6 gm. of hemoglobin, blood nonprotein nitrogen 34 mg. percent, and total serum protein 7.28 gm. percent.

Clinical course.—Two weeks later the patient was given three injections of iodopolysaccharide. Dilaudid and phenobarbital were given as preliminary medication before the first injection. The reaction following the injection was severe, although the dose was only 5 micrograms. A shaking chill started 0.5 hour after injection, lasted for 0.5 hour, and was accompanied by pallor, mild dyspnea, and slight cyanosis. Her temperature rose to

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105.2° 4 hours after injection and remained above 104° until the sixth hour. During the period of rising temperature, there was considerable nausea, vomiting, and diarrhea. She was given sodium phenobarbital, but the gastrointestinal symptoms did not subside until morphine sulfate and atropine were administered.

From the sixth to the twenty-second post-injection hour, she presented a picture of severe vascular collapse. During this period the minimum blood pressure was 68/50 mm. Hg, and except for some transient responses to pressor drugs, it remained below 90 mm. Hg systolic. Between the sixth and eleventh hours, she received a total of 3.5 cc. of 1.0 percent neosynephrine and 2.8 cc. of surgical pituitrin in intermittent doses by intravenous or intramuscular route. Between the eighth and fourteenth hours she was given a total of 1,500 cc. of whole blood. From the eleventh to the twenty-fifth hour, she received neosynephrine and surgical pituitrin as a slow, intravenous drip, with frequent adjustments of the infusion rate to keep the systolic pressure at or above 80 mm. Hg. After the twenty-fifth hour, the blood pressure stabilized between 90 and 100 mm. Hg, systolic, and urine formation was resumed about the same time.

Twenty-four hours after this first injection, the patient showed the characteristic marked general debility. However, her color was better than it had been prior to injection. Herpes simplex appeared on the lips 34 hours after the injection.

During the 4 days following this reaction, the patient continued to suffer attacks of nausea, vomiting, and abdominal pain, which seemed somewhat more severe than they had been before the polysaccharide was administered. She was treated symptomatically with morphine and atropine.

The reaction following the second injection was of far less severity than the previous one. During the chill phase, the patient suffered severe back and leg pain, attacks of abdominal cramps, severe nausea, and vomiting. The gastrointestinal symptoms continued intermittently throughout the day.

The following day the patient looked and felt extremely weak. Her abdomen was slightly distended and tender, her temperature was normal, pulse rate 84 per minute, respiratory rate 24 per minute, and blood pressure 108/84 mm. Hg.

The third and last injection of iodopolysaccharide was given that morning. The reaction which followed was of somewhat less severity than on the day before, the major symptoms consisted of nausea, vomiting, abdominal cramps, low back and leg pains, and severe malaise. A transient increase in leukocyte count of 6,000 was noted 24 hours after each injection.

During the following week the patient's nausea, vomiting, abdominal pain, and distension persisted to a degree suggesting obstruction of the small bowel. At laparotomy, marked progress of the carcinomatosis was found. No point of obstruction was encountered, but the degree of involvement of bowel serosa with tumor was sufficient to explain the patient's symptoms. Edematous, necrotic, and hemorrhagic tumor was present. Several nodules of healthy-looking tumor were removed and examined microscopically. No evidence of hemorrhage or necrosis was seen in this tissue.

In view of the rapid advance of the tumor and the patient's symptoms, narcotics were used freely. During the following week the patient ran a febrile course, became progressively weaker, and died 2 weeks after the third injection of iodopolysaccharide.

Post-mortem examination.—The peritoneal cavity contained 3 l. of blood. There was a large tumor mass the size of a grapefruit, which occupied the entire pelvis. The visceral and parietal-peritoneal surfaces were studded with multiple tumor implants of various sizes. Some hemorrhagic and necrotic tumor was seen. There was neoplastic involvement of the omentum, tumor implants over the small bowel, and metastatic nodules in the liver. The thorax, lungs, and mediastinum showed no gross evidence of metastases.

The necrosis and hemorrhage seen in many of the peritoneal tumor implants were consistent with the rapid metastatic spread of the neoplasm and could not be attributed to the action of the polysaccharide.

Case 4

Diagnosis: Scirrhous carcinoma of the breast, metastatic to skin and lymph nodes.—The patient was a 67-year-old woman who had noted a mass in the left breast 6 years earlier. Six months later a radical mastectomy was performed for scirrhous breast carcinoma with axillary metastases. Three years later there was recurrence in the left supraclavicular nodes; during the course of the following year she was given X-ray treatment to that region (a total of 9,600 r). Therapy was discontinued because of the development of local radiation reaction and partial pulmonary collapse. She developed some swelling and neuritic pain of the left arm during the several months before admission, and during the latter part of this period metastatic skin nodules began to appear on the left upper chest wall.

There was a past history of hypertension and mild cardiac enlargement of at least 6 years' duration but no history of cardiac decompensation.

On admission, the patient was in a relatively good state of nutrition, complaining only of an ache in the left upper arm. There was moderate swelling of the left arm, and many small, firm, pink nodules were seen along the course of the mastectomy scar and in the skin of the left infraclavicular and supraclavicular areas. A large, matted mass was found in the left supraclavicular fossa. There was slight cardiac enlargement, bronchovesicular breath sounds in the left lower lung field posteriorly, and occasional crepitant rales. The patient was troubled from time to time with paroxysms of coughing, productive of tenaceous, mucopurulent material in moderate amounts. Her temperature was normal. The blood pressure was 180/100 mm. Hg.

Laboratory studies revealed a normal urine, an erythrocyte count of 4.7 million, hemoglobin 13.0 gm.; leukocyte count was 9,700, with 65 percent polymorphonuclear forms, 31 percent lymphocytes, and 4 percent monocytes.

Fasting blood sugar was 105 mg. percent, blood nonprotein nitrogen was 35 mg. percent, and the total protein was 7.72 gm. percent with a normal albumin-to-globulin ratio. Roentgenograms of the chest showed some atelectasis at the left base and no evidence of metastatic disease in the lungs or visualized bones.

Clinical course.—After biopsy of one of the nodules, the patient received 4 injections of iodopolysaccharide. Following each injection, there was a characteristic reaction, consisting of a chill, rise in temperature, fall in blood pressure, and marked general debility. During the period of rising temperature, the patient suffered dyspnea, nausea, and vomiting. Moderately severe low back pain occurred during the second post-injection reaction. The lowest blood pressure reached was 88/52, and at no time were there other clinical signs of shock, such as clamminess of the skin, poor capillary circulation, or depressed sensorium. Following the first 3 injections, however, it took considerably longer than 24 hours for the blood pressure to return to the initial level of 160. Six hours after the third injection, the patient went into moderate cardiac decompensation as manifested by venous distension, tenderness and enlargement of the liver, bilateral basal pulmonary rales, and increasing dyspnea. A roentgenogram showed fluid at the left costo-phrenic angle. She was digitalized at that time with 1.2 mg. of digitoxin intravenously, and the signs and symptoms of decompensation subsided during the next 12 hours. A daily maintenance dose of digitoxin was continued thereafter.

In addition to the development of congestive heart failure, the major change in the patient's physical status during the period of polysaccharide administration was the progressively accumulating debility from which there was very little recovery in the intervals between injections. There was also some increase in the edema and the neuritis-like pain of the left arm.

Urinalyses following the reactions showed no significant deviation from the normal. Her erythrocyte count and hemoglobin remained essentially unchanged during her hospital stay. Her leukocyte count was 10,450 on the day after the first injection, 20,600 on the day after the second injection (with 81 percent polymorphonuclear leukocytes), and 12,600 three days after the fourth injection. The blood nonprotein nitrogen and the serum total protein remained normal.

There was no pain, swelling, blanching, reddening, or other gross change in the metastatic skin nodules. On the contrary, there was slight progressive increase in the size of some of them, and one new small one appeared during the period of polysaccharide administration. Microscopic examination of a biopsy specimen, obtained 7 days after the fourth injection, revealed no significant change in the appearance of the tumor.

The patient's weakness persisted. The liver edge was still palpable; but it was no longer tender, and other evidence of cardiac decompensation had subsided. The blood pressure reverted to its initial level 24 hours after the fourth injection. She remained afebrile.

During the next 4.5 months the patient was seen seven times in the tumor clinic. During the first 6 weeks of this period, there was some slight recovery from the marked debility with which the post-injection reactions had left her. Thereafter, her condition became steadily worse. The edema of her arm became more and more pronounced and the pain more severe. There was steady, relatively rapid increase in the number and size of the metastatic skin nodules, which became confluent, deep, reddish purple in color, and somewhat necrotic looking as they attained larger size. They started becoming moderately painful about 1 month after discharge. She was anorexic and developed nausea and vomiting following any kind of medication. Consequently, maintenance digitalis was discontinued about 3 weeks after discharge, but it was found that her heart remained relatively well compensated on a chair- and bed-rest regime. Her food intake was very inadequate, and she became more and more malnourished. She died 2.5 months after she was last seen in the tumor clinic. The cause of death was said to be tracheal obstruction from a metastatic mass at the base of the neck. Post-mortem examination was not made.

Case 5

Diagnosis:—Undifferentiated carcinoma of the breast, metastatic to viscera and bone.—The patient, a 42-year-old woman, had first noticed a mass in her right breast 5 years previously. Four years later she became aware of increased size of the mass and retraction of the nipple. Two months after this, radical mastectomy was performed for undifferentiated carcinoma of the breast with metastases to the axillary lymph nodes and carcinomatous extension to the pectoral muscle. At that time there was no evidence of visceral or bone metastases.

Starting 3 weeks after operation and for a period of 3.5 months, she received a course of X-ray therapy to her right axilla, right supraclavicular area, and mediastinum; the total dose to each area was 4,000 r. She suffered no radiation reaction.

The first evidence of recurrent malignancy occurred 6 months after operation when she developed lumbosacral back pain. At that time roentgenograms of her lumbar spine and pelvis revealed osteoclastic metastases in the lower lumbar and upper sacral segments, in the left ilium, neck of the right femur and upper left femur, and in several of the lower ribs.

During the 3 months before admission, the patient suffered mainly from anxiety, since her back pain was mild. There was no weight loss. No further X-ray therapy was given.

Physical examination on admission revealed a well-developed and nourished, apprehensive woman. Her temperature was normal, pulse rate 96 per minute, respiratory rate 20 per minute, and blood pressure 130/70 mm. Hg. Her heart and lungs were normal. There was a well-healed skin graft at the site of the radical mastectomy with no evidence of local recurrence; there was slight tenderness upon percussion of the lumbosacral region.

Laboratory examination revealed a urine specific gravity of 1.017. Her red blood cell count was 4.05 million, hemoglobin 11.8 gm., leukocyte count 8,700, with 65 percent polymorphonuclear leukocytes, 26 percent lymphocytes,

6 percent monocytes, 2 percent eosinophils, and 1 percent basophils.

Roentgenograms of her bones revealed progression of metastases as compared with the picture seen 3 months earlier.

Clinical course.—The patient complained of recurrent pain in her left knee but was otherwise asymptomatic until the polysaccharide injections were started. Four injections were given. The third injection consisted of a small dose of unmodified polysaccharide.

The post-injection reactions followed the characteristic pattern (fig. 4 and table 2), namely, a chill, accompanied by dyspnea, nausea, vomiting, cyanosis, and a rise in temperature and fall in blood pressure. The nausea, vomiting, and dyspnea during the acute phases of the reactions were particularly severe, although there was no apparent metastatic involvement of the lungs or gastrointestinal tract. The blood pressure reached a minimum of 72/50 during the first post-injection reaction, and the skin became clammy. She was treated with several injections of neosynephrine, pituitrin, and a 500-cc. transfusion of whole blood. Her blood pressure started a sustained recovery about 24 hours after the polysaccharide infusion. Four and one-half hours after the polysaccharide injection, she suffered an extremely acute exacerbation of the pain in her left knee which troubled her thereafter with greater severity than it ever had before. Twenty-four hours after this injection, she developed extensive herpes simplex of her lips and oral mucosa, which increased in severity and extent during the next 4 days and caused her considerable painful discomfort.

The reactions following injections 2, 3, and 4 were of much less severity than that following the first injection. However, they showed the same pattern of symptoms and temperature, respiratory, and circulatory changes. There was no serious vascular collapse during these three reactions, and the blood pressure returned to the initial level within 24 hours of the start of the injection in each instance. She became afebrile less than 12 hours after each injection. However, her dyspnea was considerably out of proportion to the over-all severity of the reactions. At no time did she show significant suppression of urine formation.

The post-reaction debility was marked and increased progressively following each injection. There was no appreciable recovery from this during her hospital stay.

Urinalysis on the day after her second polysaccharide injection was normal. Her white blood count was 26,000 on the day after the first injection, and 8,400 on the day after the second injection. Her hemoglobin level did not change.

The patient was discharged on the nineteenth hospital day, afebrile, complaining of marked debility and recurrent pain in the region of the left knee.

The patient was followed in the tumor clinic. During the first 6 weeks, she had severe residual weakness and fatigue. Some improvement occurred at the end of the second month. During this time she began to have cough, dyspnea, and a sense of heaviness in the right side of her chest. Roentgenogram showed massive, pleural effusion on the right, and about 1,200 cc. of blood-tinged amber fluid was removed by thoracentesis. Fluid reaccumulated in 4 weeks. Roentgenograms of her bones 6 weeks after leaving the hospital showed increase in the size and number of the metastases. There was no gross destruction of the bones adjacent to the left knee joint.

In the third month following discharge from the hospital, the patient showed gradual deterioration by progressive loss of weight and strength. Pain in the left knee became severe, and she developed back and hip pain. She was given X-radiation to her lumbar spine and ovaries. The pain in the back subsided.

Four months after discharge, she was given intramuscular testosterone, approximately 150 mg. per week. During the following 10 weeks, the patient had relief of pain and a general feeling of well-being. Physical deterioration continued. Thoracenteses were performed three times during the interval between 5 and 8 months after discharge because of severe respiratory symptoms. These symptoms increased, she became progressively cachectic, was readmitted, and died 10 days later, 8 months after her last polysaccharide injection.

Post-mortem examination.—The subcutaneous tissues over the anterior and right lateral aspects of the chest and right upper abdomen were completely replaced by neoplastic tissue, with irregular invasion of the right upper abdominal musculature. The medial aspect of the left breast was also involved by direct extension. The right dome of the diaphragm was thickened with tumor; there was extensive involvement of the anterior mediastinum, right pleural space, with almost complete encapsulation of the right lung, which was almost totally collapsed. There were scattered metastatic nodules in the lungs and scattered, small hepatic, peritoneal, and adrenal metastases The peri-aortic lymph nodes were extensively involved with tumor. There was no evidence of old or recent hemorrhage or necrosis of tumor tissue.

Case 6

Diagnosis: Osteogenic sarcoma of left tibia with lung metastases.—The patient was a 19-year-old male who had first developed painful swelling of the upper portion of the left tibia 16 months previously. A month later roentgenograms revealed a bone tumor at the upper end of the left tibia without pulmonary metastases. A mid-thigh amputation was performed for osteogenic sarcoma with extension to the soft tissue.

The patient was asymptomatic until 12 months after operation when he developed weakness, fatigue, a nonproductive cough, and slight dyspnea. A rotengenogram of the chest was said to reveal a metastatic lesion in the lung. His symptoms increased progressively; and 3 months after their onset, he began to develop anorexia, nausea, weight loss, pain over the entire left side of the chest, and hemoptysis. During this time, left pleural effusion developed, and two chest taps each yielded about 1 l. of blood-tinged fluid.

Physical examination on admission revealed a pale, thin man who had slight dyspnea and occasional paroxysms of nonproductive cough and complained of pain over the

entire left side of the chest. His temperature was 98.6°, pulse rate 88 per minute, respiratory rate 22 per minute, and blood pressure 140/80 mm. Hg. There was a large pleural effusion on the left with mediastinal shift and crepitant rales over the right posterior lung field. Radiography revealed several round metastases in the right lung. Three of these were calcified.

Laboratory examination revealed a normal urine, an erythrocyte count of 3.59 million with 75 percent hemoglobin, a white blood count of 8,350 with 66 percent polymorphonuclear leukocytes, 27 percent lymphocytes, 5 percent monocytes, and 2 percent eosinophils. The blood nonprotein nitrogen was 23 mg. percent and the total protein 6.29 gm. percent.

Clinical course.—The patient was treated symptomatically for his chest pain, and 1,500 cc. of blood-tinged fluid was removed from the left pleural cavity. In the course of 13 days, the patient was given five injections of polysaccharide. His fourth injection was of radioactive iodopolysaccharide. (See previous publication (*9*).) Aqueous adrenal cortex extract (Upjohn) was administered as follows (intravenously): 20 cc. on the day before injection 1, 30 cc. on the day of injection 1, 40 cc. on the day of injection 2, 30 cc. on the day of injection 3, 40 cc. on the day of injection 4, and 40 cc. on the day of injection 5.

His post-injection reaction followed the usual pattern of a chill, followed by rise in temperature and fall in blood pressure. Dyspnea was marked, but other symptoms were not so severe as seen in previous patients. There was transient asthmatic wheezing. He suffered his usual chest pain but none of the severe myalgia which usually accompanied the chill. He had no nausea or vomiting following the first two injections and only a moderate amount after the last three. Although he noted some fatigue after each reaction, this symptom was unusually mild. There was slight cumulative weakness during the period of polysaccharide injections. Although the fever attained maximum levels of 102° to 105°, the blood pressure fell below 100 mm. Hg systolic only once. This consisted of a transient fall to 88/50 mm. Hg during the second post-injection reaction.

Rapid progression of the metastatic disease was evident from steady increase in the severity of the chest pain, cough, and dyspnea. There was evidence of reaccumulation of fluid which could not be easily removed because of loculation. Several rotengenograms taken at weekly intervals showed progressive increase in the size of the pulmonary metastases.

Urinalysis showed albumin on the day after the first injection. Four subsequent urines were free of albumin. His white blood count was 14,100 on the day after the first injection, 11,000 three days after the second injection, 32,600 on the day after the third injection, and 35,800 on the day after the fifth injection. Four days after the last injection, the white blood count had fallen to 6,400, and 4 days later it was 4,100. At the time the count was 35,800, there were 95 percent polymorphonuclear leukocytes (mostly band forms), 4 percent lymphocytes, and 1 percent monocytes. When the count was 4,100, there were 50 percent polymorphonuclear leukocytes, 42 percent lymphocytes, and 8 percent monocytes. Five red blood cell counts and hemoglobin determinations during the hospital stay showed no essential deviation from the admission levels. The blood nonprotein nitrogen was 27 mg. percent on the day after the fifth injection, and the serum total protein was 5.92 gm. percent.

The patient was kept comfortable with narcotics and was discharged for care at home. He deteriorated rapidly thereafter and died 1 month later, about 6 weeks after his last polysaccharide injection. Post-mortem examination was not made.

Case 7

Diagnosis: Papillary adenocarcinoma of the ovary with generalized peritoneal and thoracic metastases.—The patient, a 33-year-old woman, had first noticed swelling of the abdomen 3 months earlier. Signs of ascites were demonstrated, and an exploratory laparotomy was performed. Both ovaries were enlarged to 1.5 times normal size and studded with tumor. There was tumor invasion of the dome of the bladder and the peritoneal surface of the uterus. An indurated mass of tumor was found in the omentum, and the entire parietal peritoneum, undersurface of the liver, and gall-bladder region were studded with tumor. Both tubes and ovaries were resected. The pathologic diagnosis was papillary adenocarcinoma of the ovary.

At the time of operation there was no physical or roentgenological evidence of thoracic metastases. During the second post-operative week, the patient was given 3,200 r of X-radiation to the anterior pelvis. This had to be discontinued because of the development of an acute radiodermatitis. Three weeks before admission to the hospital for polysaccharide administration, an abdominal paracentesis was performed yielding 2.4 l. of fluid in which carcinoma cells were found. At that time, she showed physical signs of fluid at the left lung base.

Physical examination on admission revealed a pale, thin, young woman, mentally depressed and complaining of generalized abdominal discomfort and a feeling of heaviness in her left chest. Her temperature was 98.6°, pulse rate 90 per minute, respiratory rate 22 per minute, and blood pressure 110/80 mm. Hg. There were signs of moderate pleural effusion on the left, the abdomen was greatly distended with fluid, and the lower abdominal skin was deeply pigmented and moderately inflamed.

Laboratory examinations revealed a normal urine, erythrocyte count of 3.15 million, a hemoglobin of 9.57 gm., a leukocyte count of 4,600 with 85 percent polymorphonuclear forms, 10 percent lymphocytes, and 5 percent monocytes. The blood nonprotein nitrogen was 23 mg. percent, the serum total protein 4.56 gm. percent, the albumin 2.68 gm. percent, and the globulin 1.88 gm. percent.

Roentgenograms of the chest before polysaccharide administration failed to reveal metastases in the pulmonary parenchyma or in the bones.

Clinical course.—Aqueous adrenal cortex extract (30 cc.) was given intravenously each day for 9 days starting with

the day before polysaccharide injection. Three injections of polysaccharide were given by slow intravenous drip.

The reactions following all three injections were of characteristic pattern. There was a chill, rise in temperature, fall in blood pressure with parallel changes in the pulse rate. Respiratory rate curves were influenced by the frequent doses of dilaudid which had to be given. The chills and periods of rising temperature were accompanied by pallor, cyanosis, pain in the back and legs, headache, nausea, and vomiting. These acute symptoms were of approximately equal degree following the first two injections and of somewhat lesser severity following the third injection.

Although the temperature rose at least to 104° during each reaction, the peripheral vascular collapse was of significant degree only following the third injection of iodopolysaccharide. During this reaction, the blood pressure fell to a minimum of 70/20 mm. Hg, and there were slight pallor and sweating for a period of about 2 hours just before the maximum temperature was reached. She responded well to neosynephrine and surgical pituitrin administration.

The post-reaction debility was of remarkably slight degree after each of the first two iodopolysaccharide injections, and it was only after the third injection that she showed an appreciable amount of fatigue. In the interval between the first and second injections, the patient stated that she felt stronger than she had at any time since the onset of her illness. Labial herpes developed 36 hours after the first injection and progressed with each injection.

The clinical course during and after the period of polysaccharide administration was marked mainly by evidence of continued rapid progress of the malignant process. There was steady reaccumulation of fluid in the abdomen and chest. The pain in her abdomen increased in frequency and severity, anorexia and nausea were progressively greater, and malnutrition became more and more conspicuous.

Urinalysis on the day after the first injection was normal; on the day after the second injection there was albuminuria; and 3 days after the third injection the urine was again normal. Her red blood cell count and hemoglobin remained essentially unchanged. Her leukocyte count was 16,500 on the day after the first injection with 94 percent polymorphonuclear leukocytes, 2 percent lymphocytes, and 4 percent monocytes. On the day following the second injection her leukocyte count was 30,000. Four days after the third injection it was 4,400 with 89 percent polymorphonuclear leukocytes, and three days later it was 5,650 with 82 percent polymorphonuclear leukocytes. The blood nonprotein nitrogen was 20 mg. percent, and the serum total protein was 4.04 gm. percent 4 days after the third injection.

Second admission.—She was re-admitted 11 days following discharge in a state of marked cachexia, complaining of severe pain in her abdomen. Her temperature was 98.6°, pulse rate 100 per minute, respiratory rate 20 per minute, and blood pressure 100/70 mm. Hg. Pallor had increased. Her mood fluctuated between depression and euphoria. There were signs of a moderate amount of fluid at the left lung base, and the abdomen was greatly distended with fluid.

Admission urinalysis showed albuminuria, acetonuria, and the sediment contained leukocytes.

Clinical course.—The patient was given a single large injection of radioactive iodopolysaccharide, and blood specimens were taken at intervals for radioactivity measurement. (See previous publication (*9*).) She was given 5 cc. of lipoadrenal cortex extract (Upjohn) intramuscularly in the afternoon and evening of the day before injection and 5 cc. of the extract on the morning of injection. No further cortical extract was given. The reaction following the administration of iodopolysaccharide was of great severity. The symptoms, however, were obscured because the patient was semi-stuporous from the frequent injections of morphine sulfate which were given in order to keep her completely comfortable. She was in a state of moderate vascular collapse from the fourth to the twelfth hour after injection of the polysaccharide. She received frequent intramuscular injections of neosynephrine and surgical pituitrin and 1,000 cc. of plasma intravenously. During much of this time her blood pressure was unobtainable, but her skin was cold and clammy only from the eighth to tenth post-injection hours. Following recovery from shock, a moderate amount of distension of the veins of the neck, face, and forehead was noted. The patient became afebrile 12 hours later.

During the subsequent 2 days, the patient remained afebrile, and her blood pressure fluctuated between 80 and 100 mm. Hg systolic. It was necessary to administer frequent doses of morphine or dilaudid in order to control her discomfort. She was, therefore, in a drowsy condition during most of this interval. Severe hiccough developed.

As a fifth and last injection, the patient was given a massive intravenous dose of unmodified polysaccharide (3 mg.) by the single injection technique.

The symptoms of the reaction which followed were again obscured by the narcotic stupor in which it was necessary to keep the patient. The febrile reaction was somewhat greater and the peripheral vascular collapse considerably less than that following the previous injection. The lowest blood pressure was 40/10 mm. Hg, but, in spite of the fact that no pressor drugs, blood, or plasma were given, the blood pressure was always obtainable. The veins of the face, neck, and forehead became markedly distended in the fourth post-injection hour and remained so until the tenth hour. There was coldness and clamminess of the skin from the tenth to the fifteenth hour after injection. She became afebrile 25 hours after the injection, but her blood pressure did not stabilize above 80 mm. Hg systolic until after the thirty-sixth hour.

The erythrocyte count was 3.67 million, the hemoglobin 10.1 gm., the leukocyte count 24,400, and the blood nonprotein nitrogen 24 mg. percent on the day after the fourth injection. Two days after the fifth injection, the erythrocyte count was 3.25 million, the hemoglobin 9.86 gm., and the leukocyte count 22,600.

Following the period of polysaccharide administration, narcotization to the point of coma was required to control

pain. She developed pneumonia 2 days after the last polysaccharide injection and died 3 days later.

Post-mortem examination.—There were 7 l. of turbid, amber fluid in the abdomen, extensive, massive, carcinomatous implants involving most of the serosal surfaces of the abdominal viscera, varying in diameter from millimeters to inches. There was carcinomatous invasion of the omentum, matting together of several loops of small bowel, massive confluent carcinomatous implants of both domes of the diaphragm. There was 700 cc. of turbid, amber fluid in each pleural cavity, collapse of the left lung, metastatic implants along the posterior parietal pleura, in the interlobar fissure and at the apical pleura of the left lung. No grossly visible metastases were found in either lung. There was evidence of bronchopneumonia.

Microscopic examination of the tumor revealed papillary adenocarcinoma with no evidence of destruction which could be attributed to the polysaccharide. The amount of necrosis and edema appeared to be greater in the surgical specimen obtained 4 months earlier than in tumor at the time of the autopsy.

DISCUSSION

Characteristics of the Toxic Reaction Following the Intravenous Administration of Iodopolysaccharide

Toxic reactions in these 7 patients were of the same general pattern as previously observed for unmodified polysaccharide [5] (*1–3*). In every case the reaction was ushered in by a chill which began 15 to 45 minutes after the injection, lasted for about 30 minutes, and varied in intensity from mild subjective chilliness to teeth-chattering rigors. In some cases there was prodromal malaise. At the onset of the chill there were pallor, coldness of the skin, particularly the extremities, cyanosis of the lips, hands, and feet, and irregular and labored respirations. Apprehension was a constant feature. Soon afterwards there were generalized muscle aches, felt particularly in the lumbo-sacral region, with radiation down the legs. Nausea, with or without vomiting, occurred in almost every case, usually starting during the latter half of the chill period. Abdominal cramps occurred in 3 instances. Immediately after the onset of the chill, the temperature began to rise steadily, to reach a maximum level 1.5 to 7 hours after injection. The average time required to reach the peak was 3.5 hours, whether the iodopolysaccharide was administered by a rapid injection or slow drip technique.

During the chill phase and period of rising temperature, the blood pressure and the pulse and respiratory rates fluctuated greatly, chiefly at higher than the initial levels. The fluctuations in these measurements were greatest in those reactions in which the rigors were most severe.

Coincident with the attainment of maximum temperature, the patient began to feel warm, his pallor rather abruptly gave way to a somewhat cyanotic flush, and he began to perspire profusely. A pounding headache was frequently present. The pulse became rapid and full (approximately 8 to 12 beats per minute increment for each degree rise in temperature). The respirations became deep and rapid, the rate corresponding roughly to the height of the fever, but was further increased when the vital capacity was abnormally low. Narcotics appeared to diminish these accelerating influences, chiefly with regard to respiration. The myalgias were no longer present, and the nausea and vomiting subsided over the next few hours. Persistence of nausea and vomiting occurred in patients with peritoneal metastases or other gastrointestinal involvement with tumor but occasionally did so in the absence of apparent local cause.

The temperature remained at or near maximum levels for intervals of 0.5 to 3 hours. The slope of defervescence was less steep and more irregular than the curve of rising fever. In 50 percent of the reactions, after a significant drop in temperature had occurred, there was a secondary rise which in some instances equalled or surpassed the peak of the original temperature elevation. In a few instances, further minor temperature elevations interrupted the downward slope of the defervescence curve before a sustained normal level was reached (figs. 1, 2, and 3). The secondary temperature rise was heralded by an abrupt replacement of the cutaneous flush with pallor, cessation of the feeling of warmth, and occasionally an elevation in blood pressure. At the peak of the secondary elevation in temperature there was a recurrence of the flush, sweating, and blood pressure drop. The picture was suggestive of a second pyrogenic reaction superimposed on the original reaction before recovery from the latter had taken place.

The blood pressure, which had risen during the chill phase, began to show a significant downward trend within 0.5 hour of the time the maximum temperature was reached. In some cases the

[5] Seligman, A. M.: Unpublished observations.

diastolic pressure at first fell faster than the systolic pressure. This agrees with a published report (*10*) of increased cardiac output during the flush phase of a pyrogenic reaction. The systolic pressure subsequently dropped out of proportion to the drop in diastolic pressure with narrowing of the pulse pressure. Minimum blood pressures were reached 2 to 16.5 hours from the start of iodopolysaccharide administration. Fluctuation in blood pressure occurred at hypotensive levels. Sustained rises in blood pressure started 8.5 to 23 hours after injection. In a fourth of the reactions, rises in blood pressure 4 to 17 hours after injection were followed by hypotension 8 to 29 hours after injection before normal tension was sustained. This is shown in figure 1. The injection of a single dose of neosynephrine in the eighth hour (fig. 1) is probably not responsible for the upward trend in blood pressure during the subsequent 4.5 hours, in the light of experience with other patients.

Results given in table 2 are tabulated under columns designated as "primary rise," "temporary recovery," "secondary rise," and "final defervescence," where the defervescence was interrupted by one or more significant exacerbations of fever. Blood-pressure changes are tabulated similarly. Where no distinct secondary changes in temperature or in blood pressure occurred, the intermediate columns were left blank.

The recrudescences of fever have also been observed with unmodified polysaccharide [6] (*2*). They have not been described in cases of other pyrogens (*10*). Although secondary changes in temperature and blood pressure may be related to the presence of tumor in these patients, no correlation with the estimated quantity of tumor can be seen (table 1).

Clinical evidence of shock, such as cold, moist skin, depressed sensorium, thready pulse, and impaired cutaneous capillary circulation, in addition to hypotension, was seen during 6 reactions in 4 of the patients. (See table 2, column headed "Additional evidence of shock.") In every instance, shock appeared within 1–2 hours of the time hypotension was greatest and persisted until sustained recovery of the blood pressure had begun. Injection of neosynephrine and surgical pituitrin produced transient blood-pressure elevations or no response at all. There was no evidence that pressor drugs or blood or plasma transfusions appreciably shortened the hypotensive period, although it was felt that these agents at least helped maintain systolic blood pressure at the critical level of 70–80 mm. Hg in some instances. Sustained blood pressures above critical levels were reached 8 to 35 hours after injection of polysaccharide. In some cases, the initial or usual blood pressure was not reached for 2–3 days.

Symptomatically, following the acute picture which characterized the period of rising temperature, the patients were relatively comfortable. They dozed frequently as a result of the combination of fatigue, hypotension, and narcotics. The exceptions occurred in cases of persisting gastrointestinal symptoms and painful metastases.

Mild paroxysms of asthmatic dyspnea were seen in 3 patients (cases 1, 4, and 6). The development of congestive heart failure following the third injection in case 4 probably was largely responsible for this symptom. There was preexisting hypertensive heart disease with cardiac enlargement in this case. Although pulmonary metastases were present in cases 1 and 6, bronchospasm with dyspnea may have been largely due to an underlying allergy.

Herpes simplex of the lips and oral mucosa appeared in all but case 2 within 24 to 36 hours after the initial iodopolysaccharide injection. The lesions progressed with subsequent injections. Similar lesions were noted with unmodified polysaccharide.[7]

Suppression of urine formation was noted in all instances in which there was appreciable and prolonged lowering of the blood pressure. Urine production was resumed regularly with the return of normal blood pressure. There were no subsequent elevations of the blood nonprotein nitrogen. Transient albuminuria was noted on the day following many of the reactions.

Elevations of the blood leukocyte count (two to four times normal) were observed 24 hours after injection. The greatest increase was in polymorphonuclear forms. Normal or subnormal levels were reached 1 to 3 days after the last injection. Erythrocyte counts and hemoglobin levels were not significantly affected by iodopolysaccharide administration.

[6] Seligman, A. M.: Unpublished observations.

[7] Seligman, A. M.: Unpublished observations.

A striking feature of the late post-injection phase was the patients' marked weakness and fatigue. With the exception of two patients who received adrenal cortical extract, the patients of the present series and those who had received unmodified polysaccharide[8] appeared and felt thoroughly debilitated and exhausted the following day. Recovery from this was slow, and its persistence and progression during a series of injections was one of the major factors limiting the number of injections given to any patient. Asthenia has been noted following other febrile reactions (*11*). The role of adrenal cortical extract in partially preventing this distressing symptom cannot be evaluated from two cases. Unpublished observations on patients treated with further modifications of polysaccharide have suggested possible value of adrenal cortical extract. However, exhaustion from reactions has been noted to be less severe in young patients who were not given the extract. Patients receiving the adrenal cortical extract appeared to have less severe hypotensive reactions, even when the febrile reaction was the same (note table 2, case 6).

Development of Polysaccharide Tolerance

The development of tolerance upon repeated injection of polysaccharide has been noted previously in animals (*5*), tumors (*5*), and man [9] (*2*). The mechanism of development of this resistance is not understood, and it has been pointed out that tolerance develops too rapidly to be explained by ordinary immunologic mechanisms (*5*). The development of resistance to the toxic action of iodopolysaccharide occurred in the present study. This was shown by a decrease in severity of the reaction on repeated injections even when the dose was increased (figs. 1, 2, and 3, and table 2). The most useful data for making these comparisons are found in the temperature and blood-pressure changes.

In every case the most severe reactions followed the first injection of polysaccharide. Furthermore, the major increase in resistance occurred between the first and second injections when they were not more than a few days apart. When 2 months intervened between injections (as in case 1, between injections 4 and 5), a partial loss of resistance occurred as indicated by the more severe reaction after injection 5 with a smaller dose. The effect was more noticeable in the blood-pressure than in the temperature changes.

In case 4, where the initial dose was unusually small (2 micrograms) and the reaction unusually mild, significant tolerance had not appeared by the time of the second injection (20 micrograms) but was apparent at the time of the third (20 micrograms).

In case 7, the increased severity of the reactions following the last three injections may have been due in part to the patient's rapid deterioration as well as to large increase in dosage (80, 300, and 3,000 micrograms). The patient recovered from each reaction, and death several days later was due to pneumonia complicating heavy narcotization.

Evidence for the development of cross-resistance to unmodified polysaccharide following injection of iodopolysaccharide is offered in cases 5 and 7.

Effect of Iodopolysaccharide Administration on Tumor

In the patients studied, there was no evidence that the clinical course of the disease was favorably affected by the administration of iodopolysaccharide. Pain or inflammation at tumor sites was not seen. Indeed, there was roentgenological, clinical, or pathologic evidence of uninterrupted progression of the disease in each instance. Gross and microscopic examination of biopsy and autopsy material, where obtained within a few days or weeks, showed no hemorrhagic or necrotic changes which could be attributed to the action of polysaccharide. Hemorrhage and necrosis were seen in some of the centers of large tumor masses where they would be expected to occur even in the absence of a specific tumor-necrotizing agent. They were not seen in small, fresh nodules which had not yet outstripped their blood supply.

The most severe reactions were seen in the three patients with the largest amount of tumor tissue (cases 1, 3, and 7). These patients had extensive intraperitoneal involvement and cachexia. They were markedly deteriorated with advanced disease, and so it is difficult to evaluate the relation of the severity of the reactions to tumor tissue mass in these seven patients.

These findings are not in disagreement with the report (*9*) of tumor-necrotizing and hemorrhagic

[8] [9] Seligman, A. M.: Unpublished observations.

phenomena in tumor-bearing mice given iodopolysaccharide. The doses that were effective in mice were about 100-200 times the initial doses given to the patients (on a weight basis). Although larger doses were eventually given some of the patients, the rapid development of tolerance reduced the possibility of significant tumor damage. This has also been observed in animal tumors (*5*). It would appear that effective clinical therapy against cancer with this agent must await sufficient reduction in toxicity to enable administration of much larger initial doses than have been heretofore possible.

SUMMARY

Iodinated tumor-necrotizing polysaccharide was given intravenously to 7 patients with metastatic cancer. The initial doses varied between 2 and 20 micrograms. The largest single dose was 3,000 micrograms. A total of 31 injections was made.

The post-injection reactions consisted mainly of a rise in body temperature and a fall in arterial blood pressure. The reactions were similar to those obtained with unmodified polysaccharide. No evidence was obtained that the iodinated polysaccharide was less toxic than the unmodified material.

Rapid development of tolerance with decrease in toxicity was observed following administration of iodopolysaccharide, similar to findings with the unmodified material. Cross-resistance between the two was also noted.

There was no clinical or pathologic evidence that injection of iodinated polysaccharide had any destructive effect on tumor tissue in the doses given to these patients.

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PULMONARY-TUMOR INDUCTION WITH ALKYLATED URETHANS[1,2]

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INTRODUCTION

It has been demonstrated (*1, 2, 3, 4*) that ethyl carbamate (urethan) increases the incidence and multiplicity of pulmonary tumors in strain A mice. A variety of other hypnotics were shown to be ineffective (*2*). Other esters of carbamic acid exhibited either slight activity or none at all (*4*). Studies of the relationships of chemical structure to carcinogenic activity against pulmonary tissue of the mouse have been extended to a series of nitrogen-alkylated derivatives of ethyl carbamate. The results are reported herein.

EXPERIMENTAL PROCEDURE AND RESULTS

The several derivatives of ethyl carbamate, NH_2-$COOC_2H_5$, used in this investigation were of the following types: R′-NH-R, $\begin{matrix} R' \\ \diagdown \\ \quad N\text{-}R \\ \diagup \\ R'' \end{matrix}$, and RNH-X-NHR, where R=-$COOC_2H_5$, R′ and R″=methyl, ethyl, n-propyl, isopropyl, n-butyl, and phenyl groups, and X=-CH_2-, -CH_2CH_2-, and -$CH(CH_3)$.

Strain A mice were used for testing each compound and were maintained on the National Cancer Institute stock diet (*5*) and water. Since no sex difference in susceptibility to the carcinogenic action of urethan has been established,[3] the experimental groups consisted of approximately equal numbers of male and female mice. Healthy animals, 10 to 12 weeks of age, were injected intraperitoneally with a solution of the compound under investigation. Injections were given once a week for 13 weeks. Two to three weeks after the final injection, when the mice were 6 months of age, they were killed and examined for lung tumors in the manner previously described (*2, 3*). Differences in technique in the several experiments, necessitated by differences in tolerance and solubility, together with the results obtained, are shown in table 1. The incidence of lung tumors in 6-month-old control mice was also determined.

DISCUSSION

The data in table 1 demonstrate that alkylated urethans are much inferior to the parent ethyl carbamate in ability to induce pulmonary tumors in strain A mice. Decreases in activities were, however, less pronounced than those observed in the ester homologues of urethan (*4*). At the dosage levels employed the tumor incidence in each of the groups of mice injected with mono- and dimethyl, mono- and diethyl, mono- and di-n-propyl, and mono- and diisopropyl urethans was 80 percent or more. Only the butyl and phenyl compounds failed to elicit significant increases in the proportion of tumor-bearing animals. On the other hand, the mean number of tumors per mouse fell off 90 percent following alkylation of urethan with a single methyl group. The dimethyl compound was even less active. With the exception of the N-isopropyl compound the activity of the singly alkylated urethans tended to decrease as the length of the alkyl group increased. Corresponding diminutions in potency were observed in the respective dialkylated derivatives. Without exception, each mono-alkyl derivative elicited a larger average number of tumors than did the corresponding doubly substituted urethan.

One can only speculate as to the mechanism whereby urethan, or any of its derivatives of lesser activity, initiate the development of lung tumors. The singularly high activity of urethan, per se, suggests that the intact molecule, rather than a metabolite, precipitates the oncogenic process. It is improbable that the degradation of ethyl N-methylcarbamate, for example, differs in general from that of the nonalkylated ethyl carbamate. The former should have exhibited a potency comparable to the latter if the oncogenic process is initiated by a metabolite common to the two compounds. If one assumes that urethan exerts its effect as an intact molecule, it follows that the oncogenic action of an alkyl-urethan may be due either to the compound as such or to urethan resulting from dealkylation. The latter possibil-

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[2] Presented in part at the meetings of the American Association for Cancer Research, Chicago, May 16-17, 1947.

[3] Unpublished data.

TABLE 1.—*Pulmonary tumor induction by N-substituted urethans*

Compound tested [1]	Formula	Dose [2]	Vehicle	Number of mice		Mice with tumors	Mean no. of tumors	Relative narcosis
				Start	Finish			
		Mg.				*Percent*		
Controls					141	17	0.18	
Experimental controls	$H_2N-COOC_2H_5$	0.5	[3] W	104	102	100	76.9	Moderate.
Methyl	$CH_3NH-COOC_2H_5$	.5	W	35	34	91	6.9	Moderate to deep.
Dimethyl	$(CH_3)_2N-COOC_2H_5$	.5	W	54	53	81	2.7	Do.
Ethyl	$C_2H_5NH-COOC_2H_5$	.5	W	24	22	86	4.0	Moderate.
Diethyl	$(C_2H_5)_2N-COOC_2H_5$	[4].375	[5] T	30	29	90	2.5	Moderate to deep.
n-Propyl	$C_3H_7NH-COOC_2H_5$	.5	T	54	[6]37	100	3.7	Deep.
Di-n-propyl	$(C_3H_7)_2N-COOC_2H_5$	.5	T	40	39	95	2.3	Shallow.
Isopropyl	$(CH_3)_2CHNH-COOC_2H_5$	.5	T	41	35	100	26.6	Moderate to deep.
Diisopropyl	$\begin{matrix}(CH_3)_2CH\\ \qquad\quad\diagdown\\ \qquad\qquad N-COOC_2H_5\\ \qquad\quad\diagup\\ (CH_3)_2CH\end{matrix}$	.5	T	51	48	79	1.6	Shallow.
n-Butyl	$C_4H_9NH-COOC_2H_5$	.5	T	40	38	32	.5	Moderate to deep.
Di-n-butyl	$(C_4H_9)_2N-COOC_2H_5$	.5	T	50	49	20	.2	Shallow.
Diphenyl	$(C_6H_5)_2N-COOC_2H_5$	.5	T	48	44	16	.2	None.
Methylene diurethan	$\begin{matrix}\qquad NH-COOC_2H_5\\ CH_2\\ \qquad NH-COOC_2H_5\end{matrix}$	.5	[7] T	43	37	100	28.6	Shallow.
Ethylene diurethan	$\begin{matrix}CH_2-NH-COOC_2H_5\\ \vert\\ CH_2-NH-COOC_2H_5\end{matrix}$	.5	[7] T	49	45	98	8.2	Moderate.
Ethylidene diurethan	$\begin{matrix}\qquad NH-COOC_2H_5\\ CH_3-CH\\ \qquad NH-COOC_2H_5\end{matrix}$	.5	[7] T	50	48	100	45.5	Shallow.

[1] Indicated by substituent groups on nitrogen atom in urethan molecule.
[2] Milligrams per gram mouse per week for 13 weeks.
[3] Water.
[4] Maximum tolerated dose.
[5] Tricaprylin.
[6] All fatalities occurred during early weeks; after fourth week dose was halved and administered at 3- or 4-day intervals; all mice survived the subsequent course of injections.
[7] Finely divided suspension in tricaprylin.

ity would appear to be supported by the unexpectedly large number of lung tumors in the group of mice that had been injected with ethyl N-isopropylcarbamate. Had the relatively high activity of this compound, which might have been expected to be of the low order of activity of other mono-alkyl derivatives, been dependent upon the presence of the alkyl isopropyl group, the diisopropyl compound might have been expected to elicit more tumors than were observed.

The data resulting from the use of the three diurethans tend to support the hypothesis that the oncogenic action of alkylated urethans occurs, at least in part, as a result of preliminary dealkylation of the molecule to ethyl carbamate. One may regard a diurethan, e. g., methylene diurethan, CH_3CH_2-O-CO-NH-CH_2-NH-CO-O-CH_2CH_3, as a substituted (carbethoxy) ethyl carbamate. Of the three compounds tested, ethylidene diurethan exhibited the highest tumor-eliciting activity. This compound dissociated freely in warm aqueous solvents to acetaldehyde and urethan. It is probable that it behaves similarly in the mouse and that the large mean number of lung tumors were elicited by the liberated ethyl carbamate. Methylene diurethan was somewhat more stable in vitro and produced somewhat fewer tumors on the average. Ethylene diurethan, which in vitro gave no evidence of dissociation into the intermediates from which it was prepared, exhibited an activity

more comparable with those possessed by the mono-alkylated urethan.

The data reported herein are but suggestive of the nature of the preliminary mechanism by which alkylated urethans exert their oncogenic effect. Experimental work now in progress may result in elaboration and clarification.

SUMMARY

The ability of urethan to induce pulmonary tumors in strain A mice was sharply decreased by alkyl substitution at the nitrogen atom. Judged by the average number of tumors per mouse, the activity of ethyl N-methylcarbamate was only 10 percent of that of ethyl carbamate. In general, doubly alkylated derivatives were slightly less active than mono-alkyl compounds. With the exception of the N-isopropyl compound, activity decreased as the length of the alkyl group was increased. The diurethans tested exhibted more activity than was anticipated. The possible correlations between the structure of the various compounds and their oncogenic activity are discussed in the text.

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THE CATHEPTIC ACTIVITIES OF THE TUMORS AND TISSUES OF TUMOR-BEARING RATS [1]

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INTRODUCTION

An increased catheptic activity of the tissues of animals bearing neoplasms was reported by Maschmann and Helmert (*1*), Purr (*2*), and Rosenbohm (*3*). Recently, Maver, Dunn, and Greco (*4*) reported a study of the catheptic activities of 24 spontaneous and induced mouse neoplasms, and of certain of the tissues of the mice bearing these neoplasms. The increased catheptic activities could be correlated in some cases with histologic changes in the spleens and livers of the tumor-bearing mice. These changes in the catheptic activity indicated a systemic effect of the tumors upon the proteinase activity of the host tissues. The study reported here concerns the different systemic effects elicited by 5 rat tumors.

MATERIALS AND METHODS

Four transplanted tumors and a primary liver neoplasm were employed:

(1) A highly cellular fibrosarcoma, AC–MCA, which was induced by Dr. M. K. Barrett in the Irish strain of rats by the subcutaneous injection of methylcholanthrene.

(2) Sarcoma E-2730 which arose spontaneously in an inbred line of Marshall strain rats and has been carried by W. C. Morgan, in this laboratory.

(3) and (4). Two malignant neoplasms, carcinoma 2226 and tumor 1643 which were induced in this laboratory by Morris and Dubnik [2] in inbred Buffalo rats by the ingestion of N-acetyl-2-aminofluorene.

(5) Primary liver neoplasms induced by Dr. Julius White, of this laboratory, in Osborne-Mendel rats by the ingestion of *p*-dimethylaminoazobenzene. The morphology of these tumors has been described by White, Dalton, and Edwards (*5*), and by Opie (*6*).

The tissues of normal animals of the same strain, sex, and age as the corresponding tumor-bearing rats were used as controls.

The methods used in the histologic observations were the same as were used in a previous study of the neoplastic and normal tissues of mice (*4*).

DETERMINATION OF CATHEPTIC ACTIVITY

The catheptic activities of extracts of the tissues were determined by the method of Anson (*7*) as previously described (*4*). The activities are expressed in milligrams of tyrosine color liberated by an extract of the tissue containing 1 mg. of nitrogen.

RESULTS

PRIMARY LIVER NEOPLASMS

The catheptic activities of these rat tumors fell in the range of the most active mouse neoplasms (table 1). The liver tissue surrounding the tumors, which appeared to be free of tumor but which showed microscopically the typical alterations found after feeding this carcinogen, showed equally high catheptic activity. These tumors which varied in weight from 3.5 to 45 gm. at necropsy were often palpable from 40 to 60 days earlier. The rats had ingested the dye from 158 to 188 days. The catheptic activity of their spleens was definitely higher than that of the spleens of normal rats (table 2) and there was a twofold to threefold increase in the weights of these organs. Histologically, several of these rat spleens showed moderate to marked extramedullary hematopoiesis, chiefly erythrocytic, while the spleens of the control rats showed little or none.

TABLE 1.—*The catheptic activities of rat neoplasms*

Tumor	Mean	Range of activity
	Mg.	*Mg.*
Primary liver neoplasm	0.135	0.0926–0.266
1643	.084	.020 – .174
2226	.051	.0016– .187
E-2730	.069	.034 – .103
AC-MCA	.127	.108 – .154

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[2] The histopathologic appearance of these tumors will be described in a later publication.

TABLE 2.—*The catheptic activities of the tissues of tumor-bearing rats* [1]

Tissue	Mean activity		Range in activity		Standard deviation	Standard error of difference	Significance [2] of observed difference
	Normal	Tumor-bearing	Normal	Tumor-bearing			
TUMOR 1643							
Liver	0.035	0.069	0.020–0.049	0.038–0.102	0.017	0.009	+ +
Spleen	.156	.153	.107– .190	.087– .245	.047	.028	–
Kidney	.079	.144	.063– .112	.082– .244	.046	.027	+
Lung	.097	.143	.079– .113	.057– .251	.065	.039	–
CARCINOMA 2226							
Liver	0.035	0.053	0.020–0.049	0.025–0.108	0.017	0.008	+
Spleen	.156	.150	.107– .190	.066– .239	.041	.024	–
Kidney	.079	.105	.063– .112	.052– .165	.032	.018	–
Lung	.097	.089	.079– .113	.053– .162	.027	.015	–
PRIMARY LIVER NEOPLASM							
Liver	0.044	0.144	0.030–0.086	0.072–0.195	0.029	0.017	+ +
Spleen	.147	.212	.129– .157	.160– .268	.027	.020	+ +
Kidney	.113	.157	.084– .126	.102– .219	.039	.024	–
Lung	.129	.121	.101– .179	.074– .188	.036	.023	–
SARCOMA E 2730							
Liver	0.075	0.071	0.064–0.092	0.053–0.108	0.016	0.011	–
Spleen	.165	.222	.123– .207	.190– .285	.045	.029	–
Kidney	.124	.147	.108– .139	.130– .174	.017	.011	–
Lung	.121	.147	.071– .174	.118– .160	.036	.024	–
FIBROSARCOMA AC-MCA							
Liver	0.068	0.082	0.048–0.086	0.063–0.100	0.011	0.005	+
Spleen	.214	.178	.182– .234	.145– .258	.039	.029	–
Kidney	.136	.153	.103– .192	.127– .186	.027	.018	–
Lung	.116	.133	.078– .163	.089– .157	.033	.022	–

[1] The activities of two or more samples of the following numbers of each tumor were studied: Tumor 1643, 11; carcinoma 2226, 21; primary liver neoplasm, 7; sarcoma E 2730, 4; and fibrosarcoma AC–MCA, 5.

[2] + + Highly significant, $P<0.01$. + Significant, $0.05>P>0.01$. – Not significant, $P>0.05$.

The statistical analysis was made by Jerome Cornfield, Division of Public Health Methods, Public Health Service.

N-ACETYL-2-AMINOFLUORENE-INDUCED NEOPLASMS

The two malignant neoplasms, 1643 and 2226, induced by the ingestion of this carcinogen differed in histology, in mode of growth on transplant, and in their systemic effects upon the catheptic activities of the tissues studied (table 2). In both tumors, the catheptic activity varied considerably with the varying degree of intercellular material and necrosis. There was a significant increase in the catheptic activities of the liver and kidneys of the rats bearing tumor 1643, while this systemic effect of carcinoma 2226 was observed only in the livers of the rats bearing it. Histologically, the spleens of the rats bearing both of these tumors showed noticeable increases in extramedullary hematopoiesis.

FIBROSARCOMA AC-MCA

The uniformly high catheptic activities of these tumor transplants corresponded to the highly cellular fibrosarcoma tissue seen histologically. All the transplants grew for 30 days and weighed from 45 to 75 gm. This tumor caused an increase in the catheptic activity of the livers of the rats which was not correlated with any observable histologic change.

SARCOMA E–2730

Although some of these tumors had grown for over 70 days and weighed more than 50 gm., their effect upon the catheptic activities of the rat tissues studied was slight. Histologically three spleens showed moderate extramedullary hema-

topoiesis, and there was leukocytic infiltration in the alveolar walls and around the blood vessels in the lungs of four rats.

DISCUSSION

The systemic effects of the rat tumors studied as seen histologically and by the increase in catheptic activity were definitely less marked than were observed with mouse tumors. In discussing the increased extramedullary hematopoiesis observed in the livers and spleens of the tumor-bearing mice (*4*), it was suggested that this nonspecific reaction might be stimulated by a general process which tends to reduce the blood hemoglobin in tumor-bearing animals. The mechanism by which this process increases the catheptic activities of some of the tissues of the host is not clear.

In earlier work (*1*, *2*, *8*), the systemic effect of neoplasms on the catheptic activity of the adjacent or distant muscle tissue was a matter of controversy. Since the catheptic activity of the muscles as determined in these experiments was low and often absent in both the tumor-bearing and the normal rats, no conclusions can be drawn from our experimental evidence.

SUMMARY

The catheptic activities of the following rat neoplasms were studied: (1) Primary neoplasms of the liver induced by ingestion of *p*-dimethylaminoazobenzene; (2) transplants of a fibrosarcoma induced by subcutaneous injection of methylcholanthrene; (3) transplants of a spontaneous tumor, sarcoma E 2730; and (4) and (5) transplants of two tumors, carcinoma 2226 and tumor 1643, which were induced by ingestion of N-acetyl-2-amino-fluorene.

The systemic effects of these neoplasms upon the catheptic activities of the tissues of rats bearing them differed. The primary liver neoplasm was associated with an increase in the catheptic activity of the liver and spleen. The livers and kidneys of rats bearing tumor 1643 and the livers of rats bearing the AC-MCA tumor also showed increased catheptic activity. The catheptic activities of the tissues of rats bearing the other tumors studied were not significantly affected.

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PROTEIN-FRACTIONATION STUDIES OF A MOUSE THYMOMA[1,2]

By E. C. Gjessing, T. N. Warren,[3] and Curtis Floyd, *Biochemical Laboratory, University of Virginia, Charlottesville, Va.*

This paper is concerned with the procedures for the fractionation of stroma, lymphocyte nuclei, and saline-soluble proteins of a mouse lymphoma, and with the characteristics of some of the fractions. These fractionation procedures are applied also to the calf thymus.

METHODS AND RESULTS

Mouse thymoma [4] was separated into 3 main fractions (diagram 1). The tissue was cooled to 0° immediately after removal, and fractionation was begun as soon as possible. The tumors were ground in a small meat grinder, and the tissue was suspended in 10 times its weight of 0.85 percent of saline (pH 6.8) and stirred gently for 30 minutes at 0°. The suspension was filtered through poplin cloth (25 threads per centimeter). This process was repeated twice in order to remove practically all tumor cells. The stroma retained the endothelial and reticular fat cells. The filtrate was centrifuged at 1,000–2,000 r. p. m. for 10 minutes at 0° to pack the nuclei. The supernatant containing the saline-soluble components was turbid.

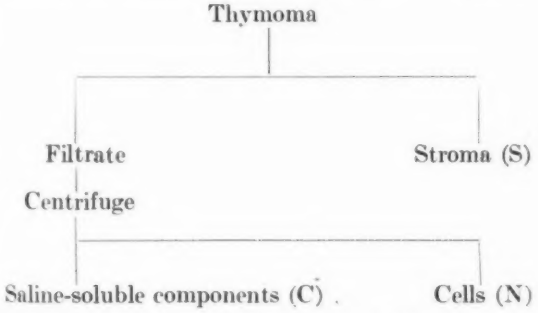

Diagram 1.—Major fractionation of thymoma.

Stroma

Two saline-insoluble fractions consisted of large, nonfilterable strands (S–I) and filterable, particulate matter (S–II). An appreciable precipitate (S–III) was obtained from the clarified supernatant at a NaCl concentration of 15 percent. By adding ethanol to supernatant S–III, a large precipitate (S–IV) was obtained. Both fractions S–III and S–IV were rich in nucleoprotein, but their solutions were not viscous.

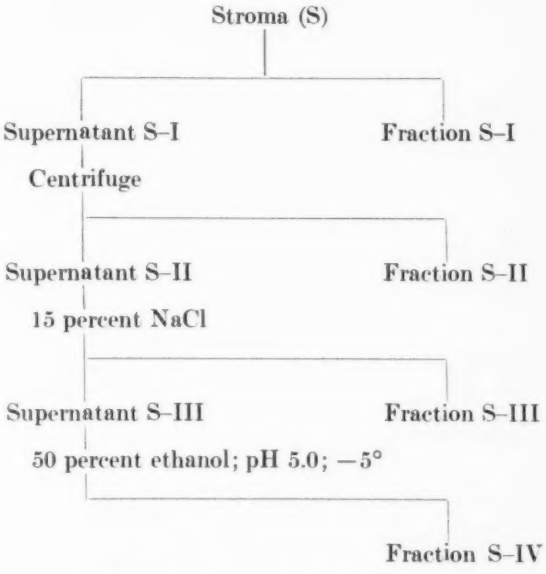

Diagram 2.—Fractionation of stroma (S).

The stroma was suspended in five times the thymoma weight of water and homogenized in a Waring Blendor at maximum speed in the cold for two 3-minute intervals. The mixture (approximate pH 6.8) was allowed to cool between extractions. This mixture was stirred for 18–24 hours in the cold room and strained through poplin cloth. The strands of connective tissue adhering to the stirring rod were removed, and the remaining fibrous material was separated by straining through cloth (S–I). The precipitate obtained by centrifuging supernatant S–I at 3,000 r. p. m. for 15 minutes at 0° is S–II. To supernatant S–II, sufficient solid NaCl was added to bring the concentration to 15 percent. This mixture was stirred rapidly at 0° for 2 hours and

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[2] This investigation was aided by grants from the medical division of the Chemical Corps, and from the National Cancer Institute of the National Institute of Health, Public Health Service.

[3] Postdoctorate research fellow, Public Health Service.

[4] This tumor was originally obtained through Dr. A. J. Dalton, of the National Cancer Institute, and was grown in dba, subline 212, mice. Details as to the type of tumor, diet on which the animals were kept, and other pertinent data are given in the following paper by Warren and Chanutin.

allowed to stand 12–18 hours. A buff-colored precipitate (S–III) was collected by centrifuging at 5,000 r. p. m. for 30 minutes. Fraction S–III was washed with 50 percent ethanol at 0° to remove NaCl. Supernatant S–III was adjusted to 50 percent ethanol with 95 percent ethanol, and the pH adjusted to 5.0 with an acetate buffer (ionic strength 0.4) of pH 4.0. After settling for 2 hours at −5°, the precipitate (S–IV) was separated by centrifuging at 5,000 r. p. m. (−5°). This fraction was washed with 50 percent ethanol to remove NaCl.

Nuclei

The nuclei of the lymphocytes which were shaken out of the thymoma by gentle stirring in saline were practically free of cytoplasm on microscopic examination. The macerated nuclei were extracted thoroughly with 0.14M NaCl, thus yielding saline-soluble and saline-insoluble fractions. The insoluble precipitate (N–I) consisted of fine threads which had an appearance similar to those described by Mirsky and Pollister (*1*). This fraction was suspended in 4M NaCl, and a heavy precipitate (N–I,1) and a water-clear viscous supernatant were obtained. This procedure was sufficient to dissolve all the nucleic acid and at the same time appeared to precipitate all the protein associated with the nucleoprotein. In contrast, similar treatment of calf thymus lymphocytes did not yield an insoluble precipitate.

In two runs, 15 and 17 percent of the total nitrogen of fraction N–I,1 was soluble in dilute HCl and was assumed to be histone. The remaining residue was insoluble in H_2O and 0.85 percent NaCl and was not entirely soluble in 0.1N NaOH. It gave positive tests for tryptophane and tyrosine. The corresponding protein which could be obtained from calf thymus by the method of Sevag, Lackman, and Smolens (*2*) was completely soluble in dilute HCl.

The saline-soluble proteins of the nuclei were fractionated to yield two fractions: Fraction N–II, obtained by isoelectric precipitation at very low ionic strengths, was relatively insoluble and showed specific absorption at 260 mμ. Fraction N–III showed no spectrophotometric evidence of nucleic acid and yielded absorption spectra typical for proteins. It was readily soluble in saline but insoluble in dilute HCl.

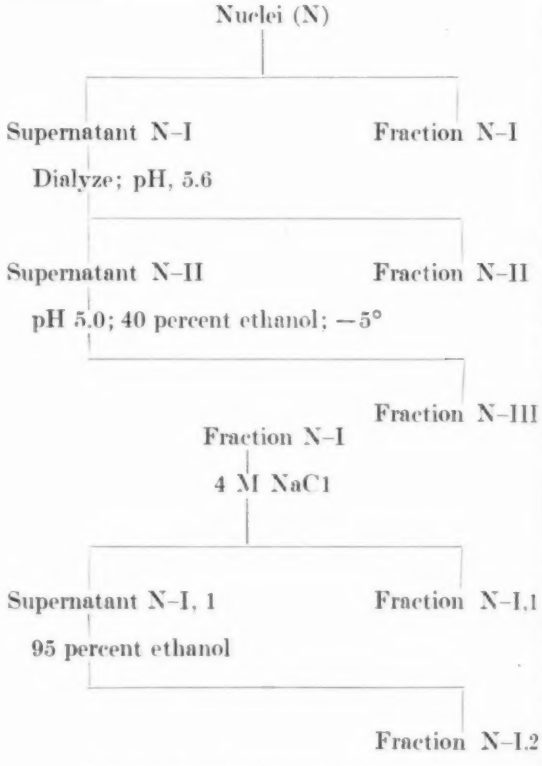

Diagram 3.—Fractionation of nuclei (N).

The tumor-cell nuclei were broken up in a Waring Blendor (0°) in the presence of 0.85-percent saline, which was equivalent to twice the weight of original thymoma tissue. The suspension was frozen at −15° to −20°, thawed in the cold room at 5°, and stirred again in the Waring Blendor at maximum speed for 3 minutes. A precipitate (N-I) was removed by centrifugation, and washed twice in the Waring Blendor with cold water. The supernatant N-I was dialyzed against tap water (5°) for 72 hours and the pH adjusted to 5.6 by addition of a few drops of a pH 4.0 acetate buffer. A precipitate (N-II) settled out in the cold room after standing for 4 hours and was removed by centrifuging at 3,000 r. p. m. for 15 minutes. Supernatant N-II was adjusted to pH 5.0 and ethanol concentration of 40 percent by

addition of 95 percent ethanol and fraction N-III was precipitated and removed by centrifuging.

Fraction N-I was mixed in the Waring Blendor at maximum speed with five times the original thymoma weight of 4 M NaCl for 2 minutes (0°). The mixture was allowed to stand in the cold room for 12–24 hours. The heavy precipitate that settled out was removed by centrifuging at 13,000 r. p. m. in a Sorvall SS-1 centrifuge for 30 minutes. This fraction was washed in the Waring Blendor with three times the original weight of thymoma tissue of 50 percent ethanol at −5° in order to wash out the NaCl. The precipitate was removed by centrifuging at 3,000 r. p. m. for 15 minutes at 0°.

Supernatant N-I,1 was added slowly to twice its volume of 95 percent ethanol (−5°). Nucleic acid was precipitated and collected on a stirring rod. The stringy precipitate was shredded with the forceps in 75 percent ethanol and stirred gently. This washing was repeated twice and the precipitate collected by centrifuging. The nucleic acid was dissolved in twice the original tissue weight of distilled water and reprecipitated by pouring it into four times its volume of 95 percent ethanol (−5°). This fraction was designated N-I,2.

Saline-Soluble Fraction

The saline-soluble solution was turbid because of the presence of a large number of microscopic particles. This particulate matter, which was removed by centrifuging at high speeds, was insoluble in saline. A small amount of material having a specific absorption at 260 mμ was lost after dialyzing the supernatant C-I.

Fraction C-II, a large fraction obtained by isoelectric precipitation at low ionic strength, yielded a turbid solution in saline. It was possible to obtain two fractions from supernatant C-II by first adjusting the solution to pH 5.0 and 24 percent ethanol and subsequently adjusting the supernatant to 40 percent ethanol concentration. On account of the small yields, they were both precipitated at a concentration of 40 percent ethanol at pH 5.0, and this fraction was designated as C-III. This precipitate was readily soluble in saline and yielded clear solutions. Fraction C-II showed a specific absorption at 260 mμ indicating the presence of appreciable amounts of nucleic acid. The presence of nucleic acid in fraction C-III is questionable according to spectrophotometric measurements.

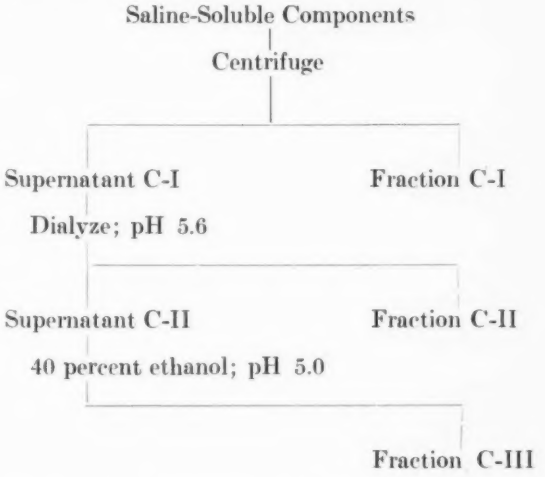

Diagram 4.—Fractionation of saline-soluble components.

The solution of the saline-soluble components was centrifuged at 13,000 r. p. m. for 1 hour at 0° in a Sorvall SS–1 centrifuge.

Supernatant C-I was dialyzed in running tap water at 5° for 72 hours and the pH adjusted to 5.6 with acetate buffer. After standing in the cold room for 4 hours, the heavy precipitate (C-II) that formed was removed by centrifuging at 3,000 r. p. m. for 30 minutes at 0°.

Supernatant C-II was adjusted to pH 5.0 with acetate buffer and the ethanol concentration brought to 40 percent with 95 percent ethanol. The mixture was maintained at −6° for at least 8 hours and the resulting precipitate (C-III) removed by centrifuging at 3,000 r. p. m. for 30 minutes at −5°.

Percentage Distribution of Thymoma Fraction

Data are shown in table 1 for the mean percentage values for the dry fractions of 14-day-old and 20-day-old thymomas. The dry weight of each fraction was determined, and the values shown represent the percentage of the total of all dried fractions. The individual variations for each fraction in the nonnecrotic and necrotic tumors are large, but these mean values give an approximation of their relative distribution. It can be seen that fractions S-IV and C-II account for almost one-half of the thymoma weight.

TABLE 1.—*Mean percentage yields of dried fractions of thymomas*

Fraction	14-day-old nonnecrotic tissue (4 groups)	20-day-old necrotic tissue (4 groups)
	Percent	*Percent*
S-I	3.0	2.6
S-II	8.9	9.4
S-III	8.5	12.4
S-IV	26.5	21.2
N-I, 1	9.2	9.6
N-I, 2	3.2	5.5
N-II	4.7	5.9
N-III	.5	.7
C-I	5.5	4.5
C-II	22.1	21.8
C-III	7.9	6.4

DISCUSSION

An extremely viscous solution is obtained whenever whole thymus or thymoma is extracted with 4M NaCl or water. It appears that this viscosity is entirely due to the nucleic acid present in fraction N–I,2 of the nucleus. All other fractions obtained by the procedures described show practically no viscosity. The RNA/DNA ratios for fractions N–I,2 and S–IV have about the same values (*3*). The difference in the viscosities of these two fractions would indicate that the nucleic acid complex of these two components is not the same.

Stedman and Stedman (*4*) maintain that all nuclei contain nucleic acid, histone, and an acidic protein termed "chromosomin." According to the present investigations, an acid insoluble in protein is obtained from the tumor nuclei of the thymoma, which has many of the physical and chemical properties described for chromosomin. From an investigation of the lymphocyte nuclei of calf thymus, no evidence of a chromosominlike protein was found.

SUMMARY

Procedures are described for fractionating mouse lymphoma tissue which avoid drastic operations and enzymatic action. The tissue is separated into stroma, lymphocytes, and saline-soluble proteins, and these main fractions are subfractionated into 11 fractions. Some of the physical characteristics of these fractions are presented. The only fraction inhibiting marked viscosity due to nucleic acid is associated with nucleoprotein of the nucleus. The proteins of the nucleoprotein of the tumor lymphocytes and nuclear particulate material are precipitated from concentrated saline solutions. This fraction contains a small amount of histone, the remainder being saline and acid-insoluble.

A fundamental difference between the nucleoproteins of tumor lymphocytes and normal thymus lymphocytes is described.

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STUDIES ON FRACTIONS OF A MOUSE THYMOMA: EFFECT OF DIET AND INHIBITING AGENTS ON THE LIPID AND NUCLEIC ACID CONCENTRATIONS[1,2]

By T. N. Warren,[3] and Alfred Chanutin, *Biochemical Laboratory, University of Virginia, Charlottesville, Va.*[4]

INTRODUCTION

The mouse thymoma provides an excellent medium for studying the chemistry of a rapidly growing lymphoid tissue at various periods of development. In addition, tumor growth may be influenced by varying the diet of the host and by treatment with nitrogen mustards (*1*, *2*) or urethan (*3*) which have specific inhibitory effects on lymphocytes.

A chemical pattern of the thymoma may be obtained by utilizing procedures developed in this laboratory (*4*) for fractionating the stroma, lymphocyte nuclei, and saline-soluble protein. Although such a pattern serves only as a rough approximation of the composition of the tissue components, it is believed that this type of approach has a great many possibilities.

The present report includes a study of the lipids and nucleic acid concentrations in the thymoma fractions of mice fed a variety of diets and treated with a nitrogen mustard and with urethan. The data indicate that the changes in the growth of the tumor do not affect the chemical patterns of the thymoma fractions.

MATERIALS AND METHODS

The thymoma used in these experiments is a large, rapidly growing type of lymphosarcoma, which originated spontaneously in a dba mouse of subline 212 at the National Cancer Institute.[5] Healthy nonnecrotic tissue from 14- to 17-day-old tumors was transplanted into dba mice, subline 212 (45 to 90 days old) obtained from the Jackson Memorial Laboratory. The mice were kept on a stock diet of Purina checkers and Gaines meal at least 2 weeks before transplantation. A tumor was dissected from a stock mouse, and a portion several millimeters in length was introduced subcutaneously, by means of a trocar, into the right axilla of the unanesthetized mouse.

The transplanted tumors are perceptible nodules on the tenth day and are about 1 cm. in diameter by the fourteenth day. On the twentieth day they appear to be as large as the body of the mouse. Fourteen-day tumors are firm and nonnecrotic, while the 20-day tumors are soft and necrotic. The mice become listless, and a number begin to die on the twentieth day, at which time they have a progressive lymphatic leukemia.

Microscopically, the nonnecrotic thymoma is a mass of tumor cells resembling large lymphocytes loosely held together by a very vascular reticuloendothelial network. It closely resembles the thymus gland but has no structural organization. In sections of 20-day tumors, large areas of necrosis can be seen, particularly near the center of the tumor. Tumor cells in many and various stages of activity and decomposition can be seen.

Groups of mice were killed by cervical fracture after 14 or 20 days. The tumors were dissected free, and the pooled tissues were kept cold while being collected. Fractionation of the tumors was begun immediately. Sufficient tissue (70–100 gm.) for study of 14-day tumors was obtained from 60 mice while an adequate amount of tissue for 20-day-old tumors was collected from 30 mice.

Urethan and methyl bis(β-chloroethyl) amine HCl (HN2) were used as inhibitory agents of thymoma growth. These agents were dissolved in distilled water immediately before injection. In one group of mice, urethan (0.5 mg./gram body weight) was injected intraperitoneally each day from the tenth to the fifteenth day, and in another group from the fourteenth to the nineteenth day (*5*). HN2 (1 mg./kilogram body weight) was injected subcutaneously on the left side of the abdomen on the fourteenth and seventeenth days. These animals were kept on the stock diet and were killed on the twentieth day.

One group of animals was fed the following synthetic diet: 25 percent casein, 2 percent agar-agar, 4 percent salt mixture No. 12 (*6*), 63.5 per-

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[2] This investigation was aided by a research grant from the National Cancer Institute, National Institute of Health, Public Health Service.
[3] Postdoctorate research fellow, Public Health Service.
[4] The authors are indebted to Jean D. Warren for technical assistance.
[5] The authors are indebted to Dr. A. J. Dalton for furnishing the stock animals carrying this tumor.

cent sucrose, 1.4 percent Mazola, 0.6 percent Natola, 0.3 percent mixed tocopherols, and 3 percent vitamin B mixture fortified with vitamin K (*7*). This group of mice was killed on the twentieth day. A choline-free, 10-percent casein diet, similar in composition to the foregoing, was prepared and fed to a group of mice which was killed after 14 days. Control animals were fed the stock diet and killed at 14 and 20 days, respectively.

CHEMICAL ANALYSES

The thymoma fractions and an aliquot of the whole thymoma were dried in vacuo over P_2O_5 in a pyrex desiccator at about $-10°$ C., in order to prevent putrefaction and enzyme action. The material was then pulverized and dried to constant weight at room temperature. All chemical analyses were made in duplicate.

Lipids

The dried material was extracted in an extraction thimble with 50 ml. absolute alcohol for 6 hours under a reflux, followed by a second extraction overnight with 50 ml. absolute ethanol-ether (1:1) mixture. The extracts were combined, and total cholesterol was determined in aliquots, according to Sperry and Brand (*8*). An aliquot of the lipid extract was evaporated, and the residue immediately extracted with petroleum ether. This extract served for the determination of total lipid carbon, according to Van Slyke and Folch (*9*), and of phosphorus, according to the procedures of King (*10*) as modified by Allen (*11*). The following conversion factors were used: ether-soluble P $\times$ 23.7 = phospholipid; phospholipid $\times$ 0.647 = phospholipid C; and cholesterol $\times$ 0.839 = chloesterol C. The combined cholesterol and phospholipid are considered to represent the essential lipid. "Neutral fat" carbon was obtained by subtracting the essential lipid carbon from the total lipid carbon.

Nucleic Acids

Nucleic acid was determined by a modification of the procedures introduced by Schmidt and Thannhauser (*12*) and Schneider (*13*). In order to remove the acid-soluble phosphorus compounds, 150–200 mg. of the dried lipid-free material was mixed with 10 ml. of ice-cold 10 percent trichloracetic acid in a large centrifuge tube, and the contents were stirred vigorously with a glass rod in the cold. This treatment was of importance in obtaining a well-packed precipitate. The solution was centrifuged, and the precipitate washed twice with ice-cold 1 percent trichloracetic acid solution. The residue was washed with water, with 95 percent ethanol, and finally with absolute ethanol-ether (1:1) mixture.

After thorough drying in the desiccator, a portion of the residue was incubated with 10 ml. of 1N KOH for about 15 hours at 37° C. (*12*) in order to eliminate the ribonucleic acid (RNA). Desoxyribonucleic acid (DNA) was precipitated from 3 ml. of the KOH solution by adding 3 ml. of an acid mixture (1 part 6N HCl and 4 parts 5 percent trichloracetic acid). After standing for 20 minutes, the precipitate was collected by centrifugation.

Spectrophotometric determinations for nucleic acids were made on an aliquot of the acid-soluble, P-free residue and on the DNA precipitate. The materials were heated for 15 minutes at 90° C. with 10 ml. 5 percent trichloracetic acid, cooled, and centrifuged. A 1-ml. aliquot of the supernatant and 1 ml. of 5 percent trichloracetic acid, which served as a blank, were diluted to the same extent and determined in a Beckman spectrophotometer at 266 mμ. The readings were made at this wavelength because trichloracetic acid caused a shift in the maximum absorption spectrum to this point. By comparing the spectrophotometric densities with a standard curve obtained from a trichloracetic acid solution of sodium thymonucleate (prepared from calf thymus by the method of Hammarsten (*14*)), the total nucleic acid and DNA content, respectively, were calculated. The difference between the values of total nucleic acids and DNA represents RNA. The validity of the figures for RNA rests on the assumption that the total nucleic acid extracts of the acid-soluble, P-free residue are not accompanied by other substances having ultraviolet absorption at 266 mμ.

RESULTS

Growth of Tumor

Definite repressive effects on the growth of the tumor are noted after treating mice with urethan or HN2. In the mice injected daily with urethan from the tenth to the fifteenth day, the tumors

regress in size in all cases but begin to grow again after treatment stops. On the twentieth day the tumors are almost as large as those of the controls (table 1). In mice treated with urethan from the fourteenth to the nineteenth day and killed on the twentieth day, the tumor is comparatively small (table 1). No gross necrosis was noted in either group. Tumor growth appears to be inhibited after the second injection in both groups.

TABLE 1.—*Effect on growth of tumors in mice on various diets*

Age of tumor (days)	Experimental conditions	Average wet weight of tumor per mouse
		Milligrams
14	Stock diet	1,450
14	10 percent casein diet	1,700
20	Stock diet	3,350
20	Urethan (10th-15th day)	2,720
20	Urethan (14th-19th day)	1,600
20	HN2	1,320
20	25 percent casein diet	3,690

Administration of HN2 inhibits the growth of the tumors after the first injection on the fourteenth day, and marked regression of growth is seen after the second injection on the seventeenth day. These tumors are the smallest encountered in this series.

The tumors of mice fed the 25 percent casein diet weighed approximately the same as those of the corresponding controls on the stock diet (table 1). At the twentieth day, the mortality of the former group (14 percent) was appreciably greater than that of the controls (5 percent). This difference in mortality may be associated with the observation that there is less gross necrosis in the tumors of the animals on the 25 percent casein diet. The weights of the 14-day-old tumors of mice on the 10 percent casein, choline-free diet were slightly greater than those of the group on the stock diet.

RESULTS OF ANALYSES

Data for lipids and nucleic acid are presented in figures 1 and 2.

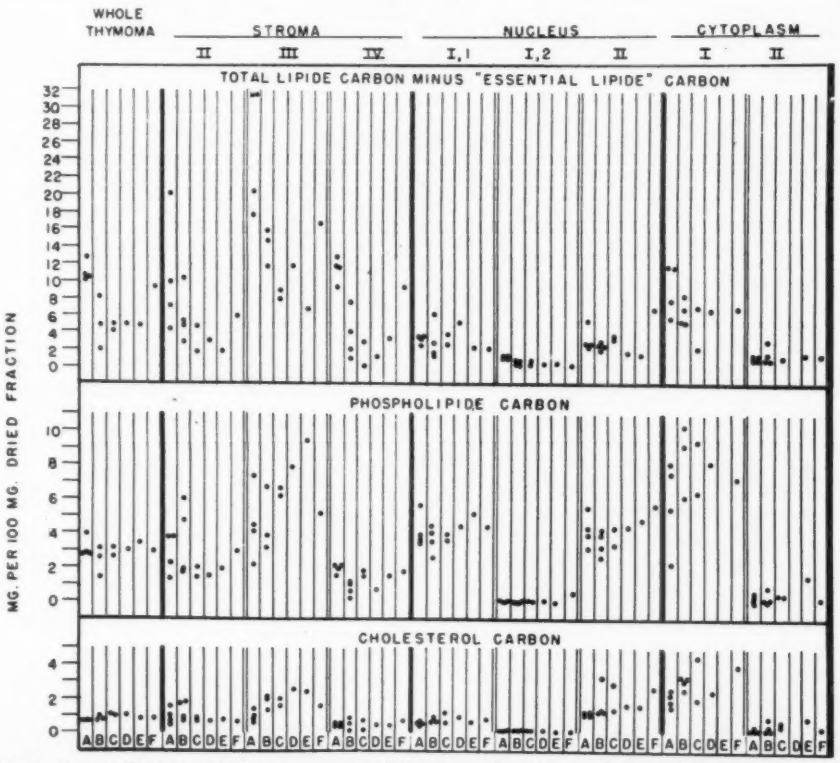

FIGURE 1.—Distribution of lipids in whole thymoma and thymoma fractions. Groups A to F were treated as indicated in the text.

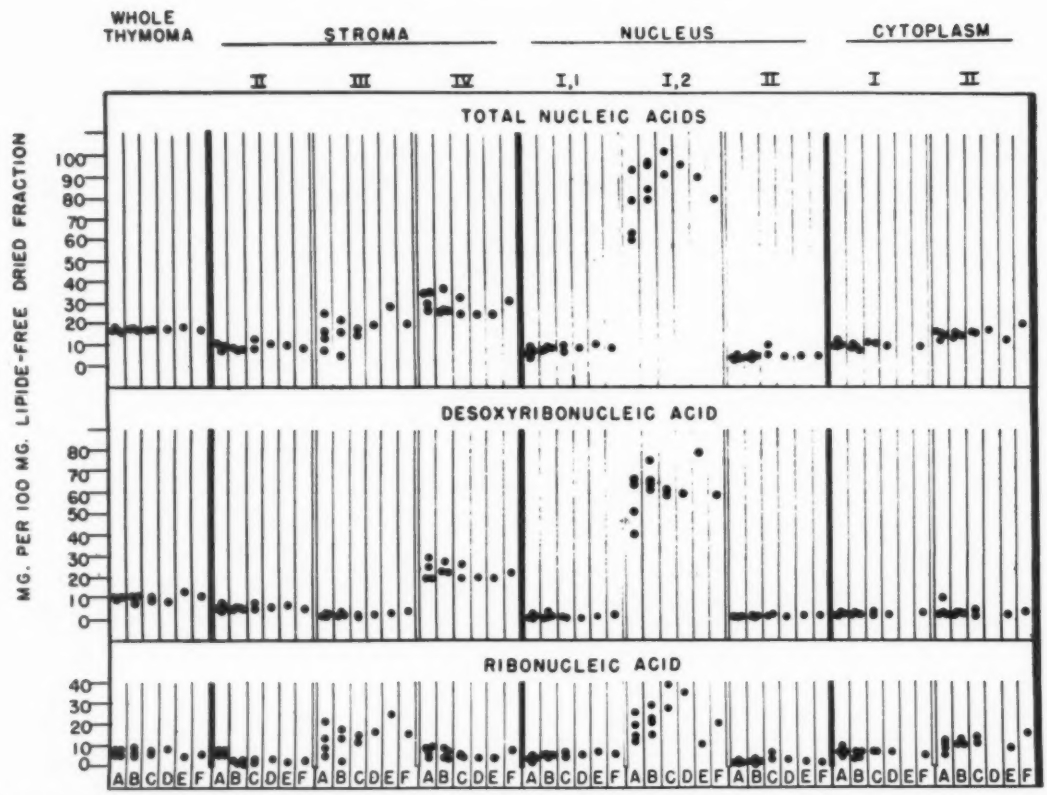

FIGURE 2.—Distribution of DNA, RNA, and total nucleic acid in whole thymoma and thymoma fractions. Groups A to F were treated as indicated in the text.

Each fraction is analyzed under six different headings, A to F, which indicate the treatment of mice:

A, 14-day-old tumors, stock diet;
B, 20-day-old tumors, stock diet;
C, 20-day-old tumors, stock diet, urethan injection;
D, 20-day-old tumors, stock diet, HN2 injection;
E, 20-day-old tumors, 25 percent casein diet; and
F, 14-day-old tumors, low protein-choline free diet.

Each fraction was analyzed for the total lipid, cholesterol, phospholipid, total nucleic acid, ribonucleic acid, and desoxyribonucleic acid. Each analysis represents a number of pooled tumors.

A total of 11 fractions was obtained, but the analyses for only 8 fractions are shown in figures 1 and 2. Fractions S-I and S-II are similar in composition and origin, and, therefore, the data for fraction S-II alone are presented. Since fractions N-III and C-III are obtained in relatively small amounts and contain traces of lipids and nucleic acid, they are omitted from the graphs.

LIPIDS (FIG. 1)

Whole thymoma.—The neutral fat concentrations are elevated in the two 14-day-old-tumor groups (A and F). The values for the 20-day-old tumors remain fairly constant, regardless of the type of treatment. The phospholipid and cholesterol concentrations are not affected by either age or treatment.

Stroma.—Data for three of the four fractions are presented. In fraction S-II, which represents connective tissue chiefly, the neutral fat, phospholipid, and cholesterol concentrations are relatively low except for the values in the 14-day and 20-day control groups. Fraction S-III is precipitated from a clarified 15 percent NaCl supernatant and probably represents a fraction of the cytoplasm of the reticular cells; the concentrations of neutral fat, phospholipid, and cholesterol are higher than those obtained for any other fraction in the stroma. Fraction S-IV is precipi-

tated out in high ethanol concentrations and is probably derived from the reticular cell nuclei; the essential lipid concentrations are relatively low and are constant in all groups while the values for neutral fat concentrations show considerable variation.

The neutral fat values are consistently the lowest in the urethan, HN2, and 25 percent casein diet groups. The significance of these findings is not clear.

Nucleus.—Fraction N-I, 1, which chiefly represents the protein of the nucleoprotein of the nucleus, is rich in phospholipids and contains small amounts of cholesterol and neutral fat. Only traces of the lipids are associated with the nucleic acid-rich fraction N-I, 2. The saline-soluble fraction N-II is comparatively rich in the essential lipids. Lipid values for whole nuclei have been presented by several investigators (*15–17*). It is clear that most of this lipid is present in two of the four fractions studied.

Cytoplasm.—The saline-soluble materials of the thymoma are present in this main fraction. Fraction C-I, separated by high-speed centrifugation, is composed of particulate materials having high concentrations of all the lipid components. Small concentrations of total lipid are present in fraction C-II, which is one of the largest subfractions. Fraction C-III contains small amounts of lipid.

The mean percentage concentrations of phospholipid and cholesterol in the six groups for all fractions are shown in table 2. The values for several of the fractions are approximations because of the large individual variations. The essential lipids of fraction N-I, 1 are particularly rich (85 percent) in phospholipids. The phospholipid: cholesterol ratios are below 1.5 in three fractions, all of which have low concentrations of the essential lipids.

TABLE 2.—*Ratio of phospholipid to cholesterol in thymoma fractions*

Fraction	Mean phospholipid value (milligrams/100 mg. dried fraction)		Mean cholesterol value (milligrams/100 mg. dried fraction)		Ratio phospholipid/cholesterol
Thymoma	[1] 2.8	(78)	[1] 0.8	(22)	3.5
S-I	1.0	(71)	.4	(29)	2.5
S-II	2.4	(73)	.9	(27)	2.7
S-III	5.5	(78)	1.6	(22)	3.4
S-IV	1.5	(79)	.4	(21)	3.8
N-I, 1	4.0	(85)	.7	(15)	5.7
N-I, 2	.1		[2]		
N-II	4.1	(71)	1.7	(29)	2.4
N-III	.5	(56)	.4	(44)	1.3
C-I	6.8	(70)	2.9	(30)	2.3
C-II	.4	(45)	.5	(55)	.8
C-III	.2	(50)	.2	(50)	1.0

[1] Figures in parentheses represent percent of essential lipids.
[2] Present in traces.

NUCLEIC ACID (FIG. 2)

Whole thymoma.—The percentage concentrations for total nucleic acid, DNA, and RNA in the six groups remain constant in all groups. The mean RNA/DNA ratio is 0.8.

Thymoma fractions.—The values for the percentage concentrations of nucleic acid for the respective fractions are fairly constant regardless of the treatment or diet of the various groups. The mean percentage values for RNA, DNA, and RNA/DNA ratios for each fraction are shown in table 3. Fraction N-I,2, which is practically pure nucleic acid, has a mean distribution of 62 percent DNA and 38 percent RNA. Every fraction contains some nucleic acid, which may be explained by the marked reactivity of this material with proteins. The effectiveness of the fractionation procedures is evident from the marked difference in the RNA/DNA ratios in fractions S-III and S-IV. The RNA is the preponderant component in all but three stroma fractions and one nucleus fraction. According to the previous paper (*4*), nucleic acid is absent or present in traces in fractions N-III and C-III.

TABLE 3.—*Ratio of ribonucleic acid to desoxyribonucleic acid in thymoma fractions*

Fraction	RNA (average milligrams/100 mg. dried lipid-free fraction)	DNA (average milligrams/100 mg. dried lipid-free fraction)	Ratio RNA/DNA
Thymoma	7.3	9.5	0.8
S-I	3.8	4.5	.8
S-II	3.3	5.4	.6
S-III	14.6	2.1	7.0
S-IV	6.8	22.2	.3
N-I, 1	6.0	1.2	5.2
N-I, 2	23.1	61.4	.4
N-II	3.4	.9	3.9
N-III	1.4	.2	6.2
C-I	7.1	2.0	3.7
C-II	11.3	2.7	4.2
C-III	2.6	.4	6.4

DISCUSSION

Brachet (*18*) and Caspersson and his associates (*19*) presented evidence to show that ribonucleic acid is concerned with protein formation in the cytoplasm. Appreciable differences in the concentrations of RNA and DNA in the different thymoma fractions are observed. The two frac-

tions of cytoplasmic origin which are obtained in largest amounts (S-III and C-II) contain the highest concentrations of RNA. These fractions may represent the greater portion of the ribonucleic acid-rich proteins which initially diffuse from the nucleolus to the outside of the nuclear membrane. The relatively high concentrations of RNA in fractions N-I,1 and N-I,2 may represent the nucleic acid extracted from the nucleoli and minute granules of the nucleus.

Organs with high physiological activity contain small amounts of RNA (*18*) and presumably have higher concentrations of phospholipids and cholesterol (*20*). These fractionation studies afford an opportunity to determine whether or not these components are associated with each other. No relationship between the concentrations of RNA or DNA with the phospholipids can be demonstrated in the various fractions.

The particulate matter of the cytoplasm of the thymoma cells is present in fraction C-I. This material contains an average of 17 percent total lipid carbon of which slightly more than one-half is composed of phospholipid and cholesterol. About 70 percent of the essential lipid is represented by phospholipid. The nucleic acid comprises about 9.0 percent of the dried fraction; RNA is found to represent 78 percent of this material. According to Claude (*21*), tissue granules in general contain 40 to 50 percent of ribonucleic acid. These values are considerably greater than those reported in this paper.

SUMMARY

Groups of mice with transplanted thymoma were fed a variety of diets and were injected with urethan and with a nitrogen mustard. Eleven fractions of the stroma, lymphocyte nuclei, and saline-soluble proteins of the thymoma were analyzed for total lipid carbon, phospholipid, cholesterol, and nucleic acid.

The growth of the thymoma was retarded by injection of urethan and nitrogen mustard.

The lipid and nucleic acid concentrations of the thymoma fractions were not appreciably affected by diet or tumor-inhibiting agents.

Phospholipids were present in greater concentrations than cholesterol in 9 of the 11 fractions. The "neutral fat" concentration of the particulate matter of the cytoplasm was relatively high; the concentrations of this constituent in the stroma were variable.

Nucleic acid was present in each fraction. The fractions of reticular and lymphocyte nuclear origin were rich in desoxyribonucleic acid. Fractions obtained from the cytoplasm contained a preponderance of ribonucleic acid.

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INFLUENCE OF AGE ON SUSCEPTIBILITY OF MICE TO THE DEVELOPMENT OF LYMPHOID TUMORS AFTER IRRADIATION [1] [2]

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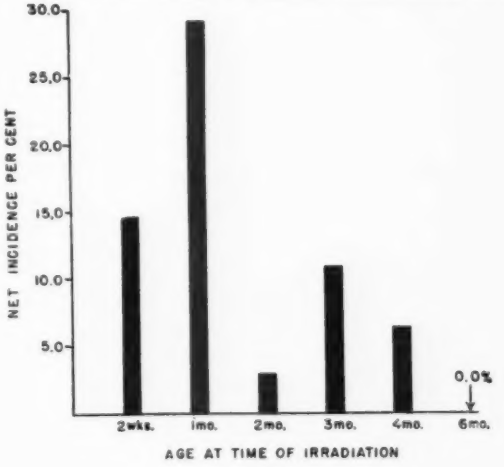
In a previous communication (*1*) it was reported that the incidence of radiation-induced lymphoid tumors in mice of strain A seemed to depend upon the age at which irradiation was administered. For ease of comparison, the results of this earlier experiment are reproduced here (fig. 1). This work has been extended to strain C57 black mice, which are considerably more susceptible to the action of X-rays, and this paper presents the confirmatory results obtained to date.

Loeb (*2*) has described unpublished experiments by Loeb and Suntzeff and by Burns and Schenken in which a similar age-dependence was demonstrated in male mice of unstated genetic background in a study of estrogen-induced mammary tumors. That this age factor may be strain-limited is indicated by the work of Cowdry and Suntzeff (*3*) working on methylcholanthrene epidermal carcinogenesis. They found that young mice of the New Buffalo strain were more susceptible than older animals, whereas no such difference existed for mice of the CBA strain.

In the present experiments, strain C57 black mice were exposed by groups to whole-body roentgen irradiation when they had reached designated ages ranging from 2 weeks to 4 months, as indicated in the table. Fractional dosage totaling 1,000 r was administered in 12 consecutive daily treatments, using a superficial therapy apparatus at 125 kv., 5 m. a., 30 cm. mouse-to-target distance, 3 mm. Al added filtration, output 42 r per minute, and a daily dose of 84 r. It was soon found that this dose produced an excessive number of deaths in the 2-week-old group, and the total dose was, therefore, reduced to 750 r for the remaining mice in this group. After irradiation, the animals were maintained on Purina Laboratory Chow pellets and water. Moribund and dead animals were autopsied, and all relevant tissues were examined histologically. The tumors were all lymphosarcomas and lymphatic leukemias identical with those described elsewhere (*1*, *4*, *5*).

The table indicates the number of mice in each age group dying with or without a lymphoid tumor during consecutive 30-day intervals after the start of irradiation. The cumulative incidence

FIG. 1. NET INCIDENCE OF LYMPHOID TUMORS BY AGE GROUPS



[1] Received for publication May 20, 1948.

[2] This investigation was initiated in the Department of Radiology, Yale University School of Medicine, New Haven, with the aid of a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

TABLE 1.—*Results of whole-body roentgen irradiation of C57 black mice*

[Expressed as: Mice dying with lymphoma/Mice dying without lymphoma]

Age group	Number of mice	Interval after start of irradiation, days—										Number of mice living
		91–120	121–150	151–180	181–210	211–240	241–270	271–300	301–330	331–360	361–390	
2 weeks	[1] 22	1/1	6/0	2/2	2/1	1/0						6
1 month	58	1/0	19/0	7/1	7/3	1/0	0/0	1/0	1/0	0/1		16
2 months	54	0/1	3/0	5/1	5/1	3/3	2/0	0/2	0/2	0/1	1/0	18
3 months	52	0/2	1/1	1/0	4/2	0/1	2/2	0/1	0/1	1/0	0/1	32
4 months	49	1/1	0/0	2/0	1/3	1/0	0/1	1/1	0/0	0/0	0/1	36

[1] Many other mice in this group died soon after irradiation before the dose was reduced to 750 r.

curves to date are presented in figure 2. It is apparent that the incidence among mice treated at 2 weeks or 1 month of age is distinctly greater, and that the preponderance of the tumors in these two groups occurs earlier, than in the older animals. The curves tend to rise rather rapidly to a plateau level which is characteristic for each age group, and the initial slopes of the curves are considerably greater for the younger mice. The numerous surviving animals in the older age groups are being maintained as long as possible to obtain further data on the terminal portions of their incidence curves.

The results demonstrate that with increasing age there is a pronounced alteration of susceptibility to the leukemogenic action of X-rays in the two mouse strains studied. Maximal susceptibility occurs at 1 month of age or earlier and is associated with a shorter mean induction time.

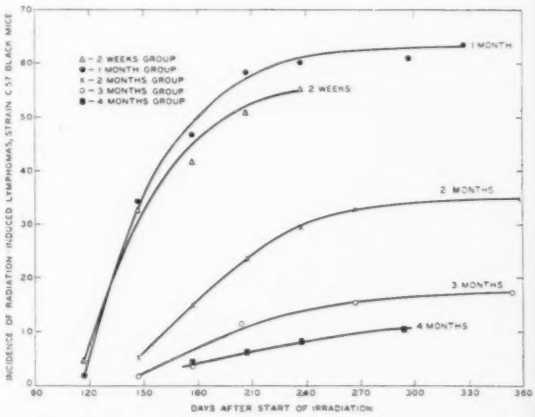


FIGURE 2.—Cumulative lymphoma-incidence curves for strain C57 black mice irradiated at the indicated ages. The 2-week-old group tolerated only 750 r, instead of 1,000 r, and is, therefore, only crudely comparable to the other groups.

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